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CHEMICAL STIMULATION OF THE CEREBRAL CORTEX

S. S. MAXWELL

From the Rudolph Spreckels Physiological Laboratory of the University of California

Received for publication September 19, 1918

Some years ago I pointed out that a broad distinction may be drawn between two classes of brain stimulants. The one class, which includes compounds like barium chloride and the calcium precipitants well known to stimulate medullated nerves, acts only on the white matter (1). These substances have no direct stimulating effect on the gray matter although Sabbatani (2) and others have shown that their presence may increase the irritability of the cortex to electrical stimulation.

The other class of chemical stimulants includes such substances as creatin, strychnine and picrotoxin. Landois (3) first pointed out that creatin and creatinin can excite the motor areas when applied directly to the exposed surface of the cerebral hemispheres. Baglioni and Magnini (4) showed later that strychnine used in the same way is a powerful excitant. On the other hand I was able to show that creatin acts on the gray matter only, since it has no effect on the white matter or on medullated nerve (5). The action of strychnine like that of creatin is confined to the gray matter.

The different effects of these two classes of chemical stimulants might be due to differences in permeability of the tissues or to differences in chemical constitution of the irritable elements, or both these factors might be involved. It is certainly fair to assume that the permeability of nerve cells and relatively naked dendrons must differ from that of medullated nerve. On the other hand the work of Moore (6) on chemical differentiation in the nervous system indicates that nerve cells themselves differ chemically, In proof of this Moore apparently has used only substances which would fall into my second class.

Since the contrast between these two classes of compounds in their stimulating action on the nervous system is so marked I was interested to discover any substance which could act in both ways. The remarkable fact that ammonium chloride does not stimulate medullated nerve while its substitution product, tetraethyl-ammonium chloride, was found by Loeb and Ewald (7) to be even more effective than sodium citrate, led me to try the latter substance on the cortex of the motor areas. I found indeed that tetraethyl-ammonium chloride is a more powerful stimulant of gray matter than creatin though less powerful than strychnine.

All my experiments were performed on rabbits. The brain surface in the region of the motor areas was laid bare by trephining, the dura was carefully removed and the localization verified by electrical stimulation. A small crystal or a small quantity of the powdered tetraethyl-ammonium chloride was then applied to the exposed surface. After a latent period of five to ten minutes muscular twitchings began in the same body regions which had responded to electrical stimulation of the cortex. The contractions gradually increased in intensity to a maximum which was usually reached in twenty or thirty minutes. The latent period was noticeably shorter and the excitation greater than when creatin was used. In order to make a more exact comparison, symmetrically situated cortical areas were exposed; powdered creatin was applied to one side and tetraethyl-ammonium chloride to the other. Without any exception the contractions resulting from the action of the tetraethyl-ammonium chloride preceded by several minutes those caused by the creatin on the opposite side.

By the method just described the stimulating effect of tetraethyl-ammonium chloride was also compared with that of strychnine. In every instance a 1 per cent solution of strychnine applied to the cortex on one side brought about the characteristic twitchings and convulsive contractions more promptly than the concentrated solution of tetraethyl-ammonium chloride applied to the other side. In a few experiments comparisons were made between tetraethyl-ammonium chloride and sodium citrate or sodium oxalate. In none of these was there any indication that the citrate or oxalate had a stimulating effect.

By the above experiments it is shown that tetraethyl-ammonium chloride unlike the other substances which stimulate medullated nerve is also a stimulant of gray matter. I hope to point out in connection with a report on other experiments the bearing of these facts on theories as to the nature of chemical stimulation.

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THE BLOOD-PRESSURE CURVE FOLLOWING AN INTRASPINAL INJECTION OF ADRENALIN

J. AUER AND S. J. MELTZER

From the Department of Physiology and Pharmacology of the Rockefeller Institute for Medical Research

Received for publication September 30, 1918

The discovery by Oliver and Schäfer that adrenal extract causes a rise of blood pressure was made by administering the extract intravenously. The blood-pressure curve which follows such an injection is characteristic. Practically without a latent period, the pressure curve rises and reaches its maximum height in a very short time. There is almost no plateau; the descent is gradual; the original pressure is attained in about five to seven minutes. Sometimes the pressure falls for a short period below the original level.

We studied at various times the character of the pressure curve which follows after diverse methods of administration of adrenalin. Among others, we studied the pressure curve which follows intraspinal injection of adrenalin in monkeys under the generally accepted supposition that absorption from the spinal canal is a slow process. The results were quite interesting. In most instances the blood-pressure curve was characteristic; the latent period was prolonged, the rise was comparatively slow, the maximum, although quite high, was only attained after several minutes and continued plateau-like for a fairly long time; the descent to normal was, comparatively speaking, very slow. These findings were embodied in a brief communication before the Society for Experimental Biology and Medicine (1).

About simultaneously with our report there appeared a brief communication of Dixon and Halliburton on the rapidity of the absorption of drugs into the cerebro-spinal fluid (2). There they stated that

A small dose of adrenalin (1 cc. of 1 in 10,000) solution, will equally rapidly produce a typical rise of arterial blood pressure, in fact almost as high as if it were injected intravenously.

In their experiments the drug was introduced through a cannula inserted into the subcerebellar cisterna. In a later article however, Dixon and Halliburton (3) make the following statement:

> Since the publication of our preliminary note, Auer and Meltzer have made some intraspinal injections of adrenalin which they expected would have no stronger action on blood pressure than that of a subcutaneous injection. They employed monkeys and injected large doses; the arterial pressure rose slowly and reached its maximum in a few minutes and remained high for a considerable time. The injections were made into the lumbar region. We have made some injections into the lumbar region but there, as we have already stated, absorption is comparatively slow and several minutes are required before any noticeable effect is observed.

Thus there is no contradiction between our observations and the results obtained by Dixon and Halliburton. In the last mentioned communication they rather confirmed our observations that when the injection of adrenalin is given in the lumbar region the absorption is comparatively slow, and several minutes are required before any noticeable effect is observed.

Dixon and Halliburton, however, fail to make any statement regarding the final intensity or the long duration of the effect. These authors were apparently interested mostly in that part of our communication which seems to contradict their statement, namely, that the absorption of adrenalin from the cerebro-spinal fluid is almost as rapid as if it were injected intravenously and confined themselves to the explanation of the seeming contradiction by the fact that our experiments were made by injections into the lumbar region while they made their injections into the subcerebellar cisterna. They were not interested in the details of the effect of intralumbar injections.

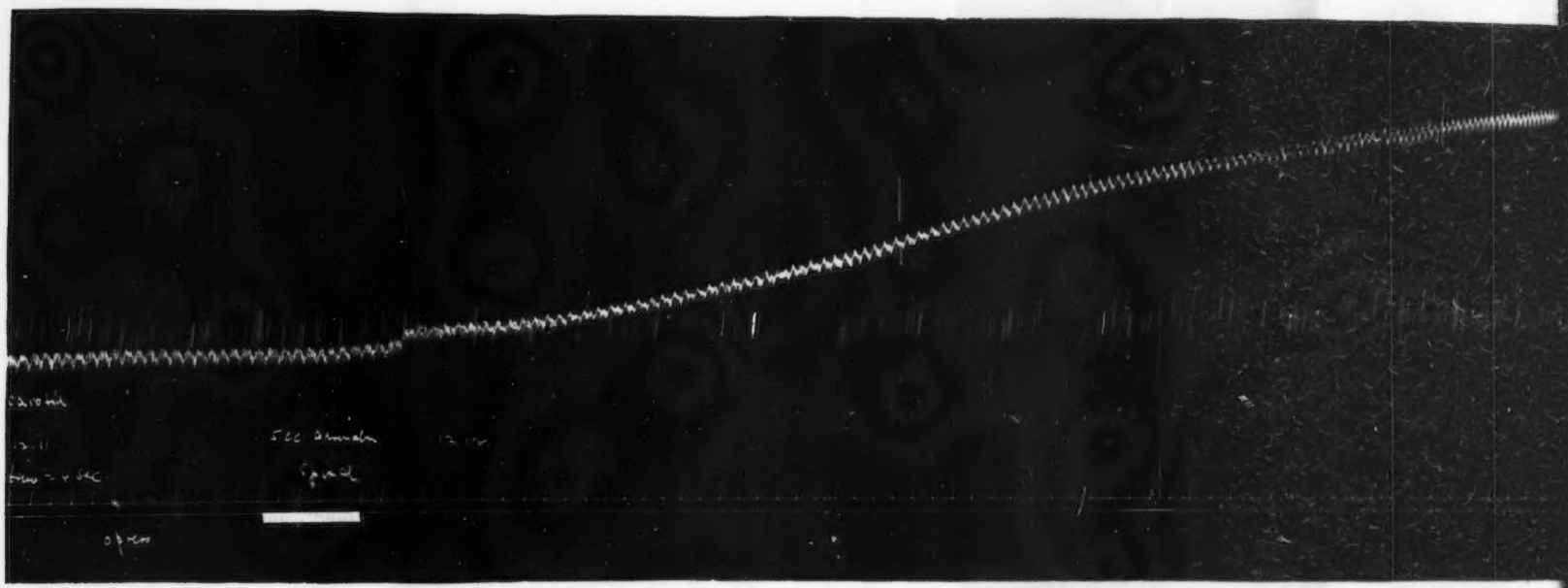
To us it seems that the most interesting part of our observations consists in the facts that the maximum of the blood-pressure rise following an intraspinal injection of adrenalin has a comparatively long plateau and the descent of the curve to the normal level is very slow; in other words, that the blood-pressure rise following an intraspinal injection of adrenalin is as a rule the longest ever observed after a single injection of adrenalin. This fact is interesting from a theoretical point of view; but it may, as we have pointed out elsewhere (4) have an important practical application. We intend therefore to describe the results of our experiments in some detail and, especially, to reproduce a most striking curve to illustrate the effects of an intraspinal injection of adrenalin. The present high price of monkeys prohibits

a repetition of our experiments on a large scale, but through the courtesy of Doctor Flexner we were enabled some time ago to make a few observations on three monkeys. These observations will be included in the following general statement.

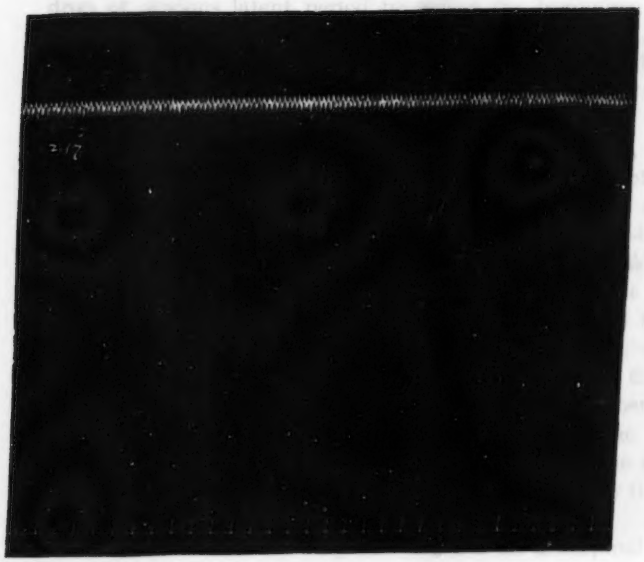
EXPERIMENTAL

Except in monkeys and human beings, it is impossible in other animals for an intraspinal injection in the lumbar region to enter the subarachnoidean space of the canal below the spinal cord; in most cases the needle enters into the cord itself and the injection is made into its tissue. In monkeys an injection below the spinal cord is frequently possible when the needle is inserted between the third and fourth or between the fourth and fifth lumbar vertebrae. However, even in monkeys the injection is not successful in every case. We have learned in the experiments with adrenalin as well as in our extensive experience with magnesium sulphate that when the escape of spinal fluid through the needle is very scant, the effect of an injection is frequently poor and not characteristic. Furthermore, on account of a venous plexus on the ventral surface of the canal and the minuteness of the cavity of the subarachnoidean space below the cord, the hypodermic needle sometimes enters into the venous plexus and the result of the injection is either that of a mere intravenous injection or, more frequently, of a mixture of an intravenous and an intraspinal injection; in the latter case the injected quantity is too small to produce a characteristic effect of an intravenous as well as of an intraspinal administration. In human beings the condition is apparently much more favorable. In the numerous injections of adrenalin made intravenously in children in the course of the epidemic of poliomyelitis (5) the effect of adrenalin upon the region of the cervical cord was unmistakable whether the quantity injected amounted only to 1 cc. or to 6 cc. for the small children afflicted with the disease.

Our experiments with the lumbar injection of adrenalin were made on ten small monkeys (macacus rhesus). The injections were as a rule made in the spaces between third and fourth or fourth and fifth lumbar vertebrae. In a few instances the injection was given in the dorsal region—with an effect generally characteristic for injections in the lumbar region. Each animal was used for several experiments after intervals of hours or several days. The smallest dose employed was 0.5 cc. and the largest 1.5 cc. of a 1: 1000 solution of adrenalin.



1A



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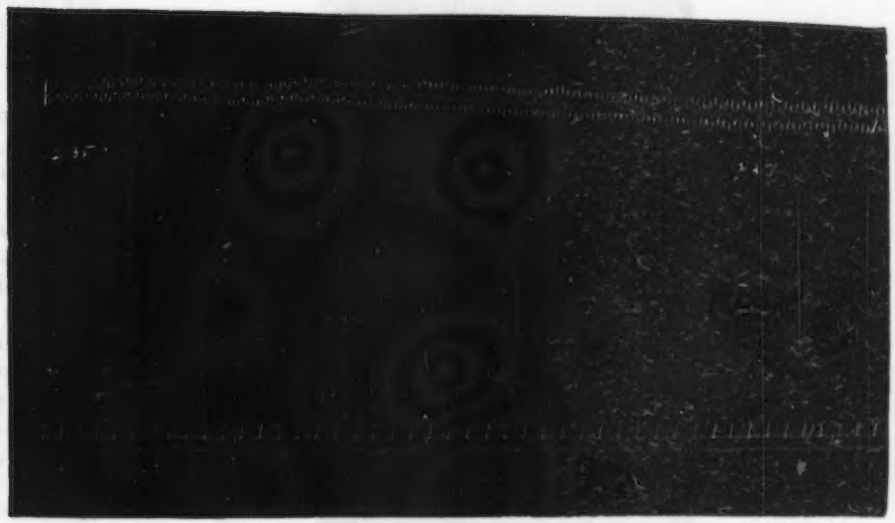
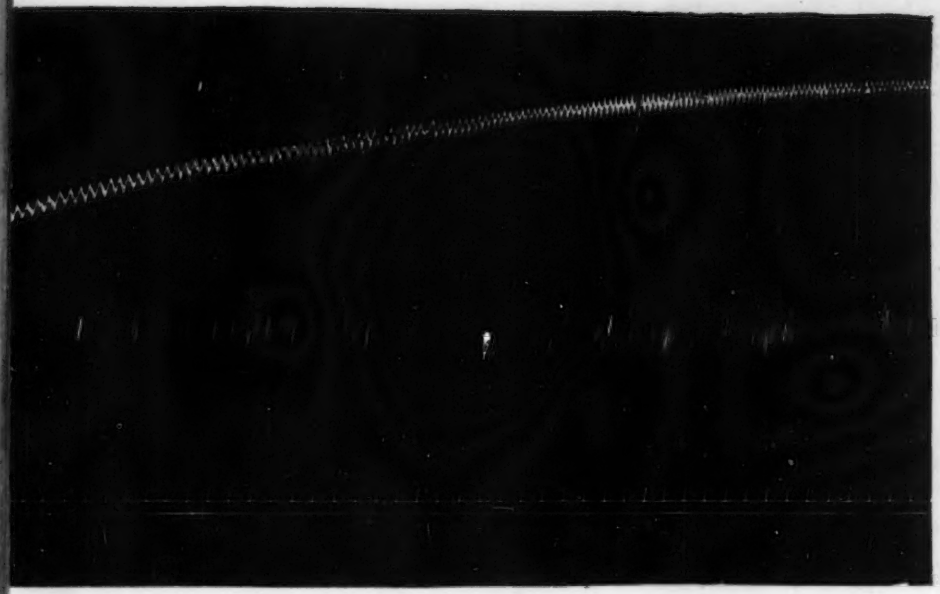
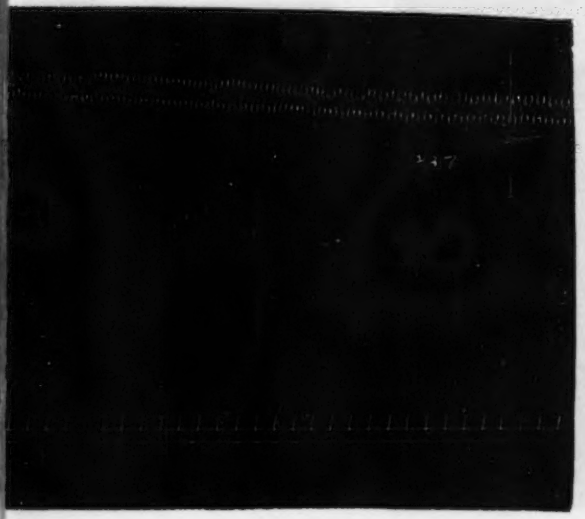


1C

Fig. 1. The four parts of figure 1 belong to a blood-pressure curve obtained from an *intraspinal* injection in the lumbar region of 1.5 cc. adrenal in a monkey. On all the figu pressure, the middle line marks the time in four seconds and is also the base line of the blood-pressure curve. On figure 1A the stimulation line carries a mark indicating t jection of adrenalin. Before the injection the blood pressure was 62 mm. After the injection the blood pressure began to rise slowly; after 30 seconds the blood pressure w it was 140 mm. Figure 1B (continuation from fig. 1A) after 5 minutes blood pressure was 170 mm.; about 7 minutes after the injection the blood pressure was 172 mm. continued for several minutes with practically very little change (plateau-like). Then it began gradually to fall. Figure 1C (the continuation of 1B) shows that about 2 pressure was still 138 mm. and the decline was going on very slowly. Figure 1D shows that at 1.10, that is, 58 minutes after the injection, the blood pressure was 120 mm. and 3 minutes after the injection, the blood pressure was still 114 mm. mercury. These figures show then that 1 hour and 3 minutes after an intraspinal injection of adrenalin the original blood pressure—62 mm. at 12.12 p.m. and 114 mm. at 1.15 p.m.





1C 1D

n the lumbar region of 1.5 cc. adrenal in a monkey. On all the figures the upper line shows the blood ve. On figure 1A the stimulation line carries a mark indicating the time and duration of the in- ssure began to rise slowly; after 30 seconds the blood pressure was only 74 mm.; after 3 minutes 7 minutes after the injection the blood pressure was 172 mm. This was the maximum, which to fall. Figure 1C (the continuation of 1B) shows that about 24 minutes after the injection the , 58 minutes after the injection, the blood pressure was 120 mm. and even at 1.14, that is, 1 hour t 1 hour and 3 minutes after an intraspinal injection of adrenalin the pressure was still high above

The character of figure 1 reproduced in this paper is typical for most of our results although we have to state that it represents the most striking result which we have obtained in our series of experiments. It was obtained January 17, 1912, from a second injection of 1.5 cc. adrenalin into the lumbar region of a male monkey; no spinal fluid was obtained at this (second) lumbar puncture. The original blood pressure was 62 mm. mercury. There was no initial drop of blood pressure following the injection, and the rise began after a latent period of eight seconds. The maximum height of 172 mm. was reached in six minutes. The maximum level presented practically a plateau of several minutes duration. The pressure fell exceedingly slowly; for one hour after the injection the blood pressure was still 48 mm. of mercury above the original level (see figs. 1 A, 1 B, 1 C and 1 D).

Figure 2 is from an intravenous injection of 1 cc. of adrenalin administered to a male monkey (2260 grams) November 8, 1917. This tracing is reproduced here for the purpose of emphasizing the contrast between intraspinal and intravenous injections of adrenalin. In this experiment the original pressure was 40 mm. mercury. There was no drop or obvious latent period preceding the beginning of the blood-pressure rise; in eight seconds the maximum of 148 mm. mercury was reached; there was no plateau. After eight minutes the blood pressure was again 47 mm. mercury, a level only slightly higher than the original blood pressure.

Without entering upon the details of our experiments, we may make the following general statement. With negligible exceptions, we may say that the blood-pressure elevation brought about by an intraspinal injection is characterized by a slow rise, by a "plateau-like" perseverance of the maximum rise, by a comparatively very slow fall to the normal level and, in general, by a long duration of the pressor effect when compared with an intravenous injection. These blood-pressure curves vary with the individual animal and its health; with the presence of fluid in the spinal cavity below the cord; with the quantity of adrenalin injected and to a degree also with the number of injections of adrenalin administered to the same animal and the length of the interval between the injections, and especially with the place of injection, for a lumbar injection is distinctly more effective than a thoracic one.

In some instances the very first effect of an intraspinal injection was an initial drop in blood pressure. The drop was usually of short duration and was followed practically in all cases by a characteristic

Fig. 2. Shows the blood-pressure curve after an *intravenous* injection of 1 cc. adrenalin into a monkey. The first line represents the pressure curve (pulse pressure very small), the second carries the time markings (4 seconds) and marks the base line while the third line shows the time of the injection of adrenalin and the washing down of adrenalin by 2 cc. of a Ringer solution. The blood pressure before the injection was only 40 mm. The injection of 1 cc. of adrenalin into the right femoral vein raised the blood pressure abruptly and the immediate injection of 2 cc. of Ringer solution, to wash out the adrenalin remaining in the cannula, brought the blood pressure to its maximum height of 148 mm.; the maximum was thus attained 20 seconds after the injection of adrenalin. About 5 minutes later the pressure fell to 64 mm. and gradually came down to a level which in this case remained slightly above the original low blood pressure.

rise. In one case after a lumbar injection of 1.5 cc. the drop was as great as 52 mm. mercury, the pressure falling from 116 to 64 mm., but it soon began to rise and in four minutes reached a height of 162 mm. mercury. In one animal however, after an injection of 1 cc. (no spinal fluid) the blood pressure slowly fell 10 mm. with no subsequent rise of pressure. This monkey was sick. However, after a second injection of 1 cc. adrenalin into the same monkey, no drop of blood pressure occurred and after a latent interval of forty seconds the pressure rose in eight minutes from 100 mm. to 138 mm.; this rise lasted twenty-two minutes. In these two injections the puncture yielded no spinal fluid. Later, in the same animal, after an injection of 1.5 cc. adrenalin, there was first a short preliminary rise followed by a drop to 86 mm. mercury lasting twenty-four seconds and then, during two minutes, the blood pressure rose to a maximum of 143 mm.; after forty-seven minutes the pressure sank again to 84 mm. In this instance the puncture had shown the presence of spinal fluid. In another animal an injection of 1 cc. caused a drop from 72 to 48 mm., that is, a drop in blood pressure of 24 mm. mercury which was not followed by any rise. However, twenty-eight minutes later, after a second injection of 1 cc. there was neither a drop of blood pressure nor was there any latent period, but in the course of three minutes after this injection the blood pressure rose from 50 mm. to 124 mm. of mercury. (Study of the further course of blood pressure in this experiment was prevented by the formation of arterial clots.)

The preceding results were observed after injections into the lumbar region. We have, however, records of a few observations on the effects of intraspinal injections when the site of application was the thoracic region of the cord. In one instance, after an injection of 1.5 cc. of adrenalin into the fifth thoracic interspace, there was no initial drop of blood pressure; a latent period of thirty-six seconds elapsed before the rise began and in two minutes the pressure rose from 54 mm. mercury to 196 mm.; after twenty-four minutes the pressure was still 100 mm. of mercury. In another animal an injection of 1 cc. of adrenalin in the fifth thoracic vertebral interspace brought an initial drop of blood pressure of 25 mm. which lasted two minutes. Then in the course of the following five minutes the blood pressure rose from 102 mm. to a maximum of 170 mm. and after fifteen minutes the blood pressure was still 134 mm. mercury. In general, however, the rise of blood pressure after intrathoracic injections of adrenalin was neither very high nor of striking duration.

CONCLUSIONS

Intraspinal injections of 1 cc. or 1.5 cc. of adrenalin in the lumbar region of monkeys cause a rise of blood pressure distinctly different in character from the curve obtained after the intravenous injection of adrenalin. The curve of blood pressure following an intraspinal injection is generally characterized by a slow rise from the original level to the maximum height; by a plateau-like duration of the maximum; by a slow fall to the blood pressure which prevailed before the injection of adrenalin. It may be stated in general that an intralumbar injection of adrenalin in monkeys causes a much more lasting effect upon the rise of blood pressure than an intravenous injection. It may perhaps be added that, as a rule, after intraspinal injections the pressure at the end of the pressor effect has not been observed to fall below the original level.

The question discussed in this paper has not been studied in human beings. While there is sufficient experience to show that an intralumbar injection of adrenalin in human beings is apparently innocuous, the effect of such an injection upon the blood pressure has not been studied properly. When we consider, as we stated before, that in human beings an intralumbar injection can be carried out with greater safety and certainty than in monkeys, the advisability of such studies in clinical physiology is quite evident.

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XVII. THE METABOLIC GRADIENT UNDERLYING COLONIC PERISTALSIS

WALTER C. ALVAREZ AND ESTHER STARKWEATHER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

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In previous studies on the stomach (1), small intestine (2) and cecum (3), we have found that there is a gradient in the CO_2 output and catalase content of the muscle from one end of the organ to the other. We came to the conclusion that the metabolic gradient thus discovered underlies and probably gives rise to the gradients of irritability, rhythmicity and latent period which in their turn probably determine the direction of peristalsis in the tube. In this paper we wish to report similar studies made upon the colon. It should be stated here for the benefit of those who may not have read the previous papers that we found the curves of catalase content and CO_2 output so nearly parallel that we feel that the catalase is a pretty good index to the metabolism of the tissue. When confronted at first by the great variations in the figures obtained in different animals of the same species we rather despaired of being able to use what seemed to be so inaccurate a method. The remarkable constancy, however, of the gradation in any one intestine has satisfied us that these determinations are trustworthy.

Technic. The method used has already been described (2). Briefly, we have made five determinations at a time, using a shaking machine. We have used rats, guinea pigs, rabbits, cats and dogs. These animals were killed by a blow on the head or by chloroform and bleeding. The segments were removed immediately. In cats and dogs the muscle could be peeled off from the mucosa but in the smaller animals the mucosa had to be scraped off. In all the tables the figures represent cubic centimeters of oxygen at room temperature and atmospheric pressure, liberated in fifteen minutes from 30 cc. of 1.5 per cent neutral hydrogen peroxid by 0.3 gm. of finely minced muscle. The figures 1 to 5 on the left hand side represent segments taken at intervals along the colon from the ileocecal sphincter to the anus.

Cat colon

																								Average
1. Upper	45.1	23.5	12.8	31.3	43.3	38.2	45.0	30.0	31.4	39.7	30.0	34.6	29.3	21.0	32.8	27.8	33.0	48.6	28.5	29.9	33.5	35.6	29.5	32.8
2.	42.3	17.9	10.0	29.1	27.5	35.1	36.5	29.9	21.4	41.5	23.5	24.0	23.1	18.9	27.8	18.1	29.0	40.0	24.6	20.4	20.3	19.1	27.8	26.5
3.	38.5	12.5	9.5	29.2	26.2	35.3	37.3	26.9	21.1	30.2	22.4	24.2	22.6	24.4	26.3	21.4	19.1	31.6	22.8	18.5	23.8	28.6	22.2	25.0
4.	29.6	12.7	9.8	32.8	31.5	40.2	43.2	29.3	21.4	31.7	24.6	29.0	29.4	25.1	17.1	19.2	18.8		26.0	19.5	27.1	29.7	22.3	25.9
5. Lower	31.1	12.3	8.4	31.8	26.1	34.5	39.1	14.5	17.3	35.1	23.0	28.1	28.0	20.8	17.6	24.7		28.0	35.6	27.3	23.4	23.2	26.7	25.4

Dog colon

														Average
1. Upper	17.9	21.1	22.2	20.2	18.0	16.5	16.6	21.6	16.4	21.2	18.6			19.1
2.	17.5	17.5	11.1	13.9	13.3	15.7	16.1	18.8	13.4	20.0	17.8			15.9
3.	15.5	15.2	16.8	17.7	14.2	15.3	12.1	14.4	14.1	17.3	17.3			15.5
4.	14.6	16.2	11.8	13.3	6.7	16.7	12.2	12.0	15.1	16.9	16.9			13.9
5. Lower	15.1	14.8	7.1	6.7	14.1	12.1	11.7	8.5	11.5	13.6	16.2			11.9

Guinea pig colon

							Average
1. Upper	47.0	47.8	45.4	46.8	51.0	48.5	47.8
2	39.2	35.0	38.7	27.6	50.9	48.8	40.0
3	44.5	43.6	31.9	29.4	40.7	31.2	36.9
4	31.8	37.8	31.5	25.3	26.5	24.5	29.6
5. Lower	37.2	37.3	26.8	18.7	20.3	27.4	27.9

Rabbit colon

								Average
1. Upper	40.0	44.6	41.4	28 1	27.9	26.4	26.0	25.0	32.3
2	33.5	31.5	37.8	23.4	22.6	21.0	18.4	20.0	25.9
3	21.7	33.7	29.6	24.7	22.5	20.9	17.8	18.6	24.1
4	27.4	33.4	30.0	20.0	21.0	20.3	18.5	23.2	24.2
5. Lower	18.6	28.4	26.2	18.7	17.6	18.8	13.0	22.8	20.5

White rat colon

							Average
1. Upper	21.4	18.6	19.4	24.3	37.0	21.1	23.9
2	20.1	18.5	20.0	20.0	35.7	20.4	22.4
3	19.7	17.4	19.8	20.0	37.3	14.7	21.5
4. Lower	12.2	12.2	8.3	11.0	23.3	12.9	13.4

Ileo-cecal junction. Some observations by Alvarez (4) suggested that the ileocecal region in rabbits has a higher rate of contraction than that of the ileum a little above. Keith (5) feels that this is an important point in connection with his discovery of what seems to be a band of nodal tissue in that region. Unfortunately we have been unable to study the catalase content of the muscle in the sacculus rotundus of the rabbit because it cannot be separated from the mass of lymphoid tissue at that point. As would be expected, this tissue has a high catalase content. The following figures from the rabbit show no definite increase of catalase in the segment next to the sacculus. In the cat and dog where pure muscle could be obtained, there was nothing peculiar about the catalase content of the terminal ileum and ileocolic sphincter.

	CAT (MUSCLE) AVERAGE OF THREE	DOG (MUSCLE) AVERAGE OF TWO
1. Ileum	29.7	19.9
2. Ileum	30.4	19.7
3. Terminal ileum	27.3	18.4
4. Sphincter	26.5	15.9
5. Colon	23.9	14.0

	RABBIT (MUSCLE AND MUCOSA) AVERAGE OF SEVEN
1. Ileum	43.9
2. Ileum	42.5
3. Terminal ileum	42.9
4. Sacculus	56.1
5. Sacculus	56.9

CO_2 estimation. Although our previous work has made us feel that we can accept the catalase content as a fair index of the metabolic rate, we thought it best at the same time to make a few estimates of the CO_2 production. We have used Haas' method, which was described fully in a previous paper (2). Briefly, weighed segments of washed gut are sealed into small test tubes containing warm Locke's solution to which a little phenolsulphonephthalein is added. The CO_2 formed in the tissue gradually acidifies and decolorizes the solution. After this decolorization has proceeded sufficiently, the tubes are graded according to color, the palest being on the left. In the following tables the figures represent parts of the colon from cecum to anus—1 being near the ileo-cecal sphincter and 5 near the anus. Sometimes two or three tubes were so nearly of the same color that they have been bracketed together.

Cat	*Rat*
3 4 5 2 1	2 1 3 4
3 1 2 5 4	1 2 3 4
5 3 1 2 4	1 2 3 4
5 1 2 3 4	2 1 3 4
1 2 3 5 4	3 2 4 1
2 4 5 3 1	2 1 3 4
3 1 2 4 5	2 1 3 4
1 2 5 3 4	1 2 4 3
1 2 5 3 4	2 1 3 4

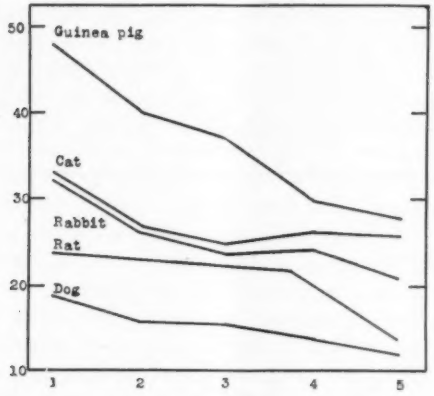

Fig. 1. Average gradient of catalase content of the colonic muscle in various animals. The ordinates represent catalase values expressed in cubic centimeters of oxygen liberated; the abscissae represent the five segments from which muscle was taken.

In some instances the gradation was brought out by titrating the solution back to its original color. The following figures represent the average number of drops of n/50 NaOH required for the different segments.

Cat

5.4 5.1 5.4 4.4 4.9

Rat

5.5 4.8 4.0 3.1

DISCUSSION

Here again in the colon we find gradients of catalase content and CO_2 production similar to those in the stomach and small intestine. When the catalase averages are plotted as in figure 1, it is seen that the gradient is most pronounced and most even in the guinea pig, rabbit and rat. It is less pronounced in the dog; and in the cat the aboral third is actually reversed. The individual curves in a number of cats were even more irregular, as can be seen in figure 2. There the only fairly constant features are the drop from the cecum to segment 2 and the rise from segment 4 to segment 5. Many of the dogs showed a similar type of curve but the average yielded a nearly straight line with a slight gradient downward to the anus.

The CO_2 estimations in the cat and rat showed gradations corresponding fairly well to those observed with the catalase in those animals. Attempts to show a gradation in rhythmicity down the colon have not been entirely successful on account of the irregular character of the contractions in excised segments. In the rabbit, however, the

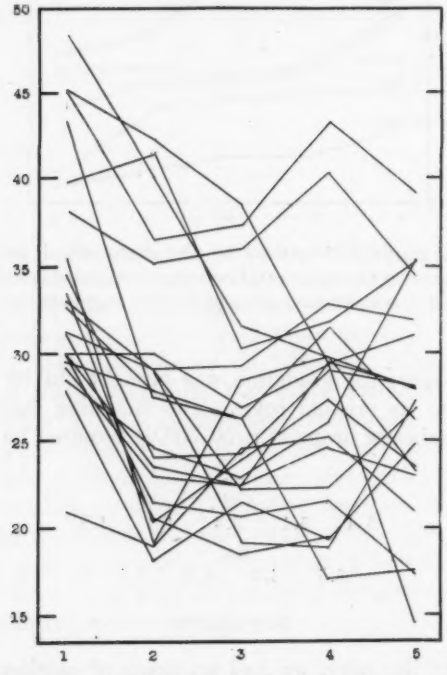

Fig. 2. To show the variations in the gradient of catalase content in the colons of individual cats. The ordinates represent catalase values and the abscissae segments along the colon.

average rate seemed definitely higher in the upper part of the colon than in the lower.

Before proceeding to a discussion of the possible significance of these findings it might first be well to review briefly what is known about colonic peristalsis. In the first place the movements are more sluggish than those of the small bowel and the neuro-muscular apparatus seems less sensitive to stimuli. Thus one of us found it almost impossible

to get any records of the colonic contractions in a woman with a fistula into the descending colon. The stimulus of blowing up the balloon sometimes gave rise to one or two contractions but after that even alternating deflation and inflation had little or no effect. Things were very different in several men with jejunal fistulae. In them the bowel was constantly contracting and pulling upon the balloon.

In man there are probably only a few large propulsive movements in the colon during the day. These occur usually after meals and often bring about a desire for defecation. The colons of rabbits, cats and dogs exposed under salt solution also remain quiet for long periods of time. We would expect then to find that the colonic neuro-muscular apparatus is different from that in the small intestine and more like that in the cecum (3). The differences that we have found in the rhythmicity, type of contraction curve, latent period and reaction to drugs (6) have all suggested that the metabolism is more sluggish in the colonic wall than it is in the wall of the small bowel. We were not surprised therefore to find the catalase content and the CO_2 output low in the colon. In the cat and dog the catalase content of the first colonic segment was often higher than that of the last ileal segment but the rest of the colon showed values lower than the lowest in the small bowel. The CO_2 output of the colon was generally much less than that of the lower ileum. A slight increase in metabolism on the lower side of the ileo-cecal sphincter might have something to do with the physiological stasis in the terminal ileum.

The striking peculiarity about peristalsis in the colon is that it is often reversed, particularly in the proximal half. Cannon (7) observed this first in the cat where the phenomenon is perhaps most marked. It has been seen in the dog, rabbit, rat, guinea pig, hedgehog and ferret (8). Retrograde transport of material in the human colon is commonly seen but the actual reverse waves are hard to demonstrate. Case (9), however, reports having seen them in 37 out of 1500 cases. As was to be expected, they were most pronounced in the presence of colonic obstruction or with spastic constipation accompanied by or due to an increased tone at the lower end of the bowel.

In view of this tendency to anastalsis it is not surprising to find that the gradient in the colon is irregular and reversed in places. In the small bowel, where katastalsis is the rule, the gradient was steep and even in all the animals. It is somewhat disappointing, however, to find in the cat the steepest and most constantly downward part of the catalase gradient on the right side near the cecum, just at the point where the reversed waves are most commonly seen.

Another interesting thing is the fact that in the herbivorous guinea pig and rabbit the gradient was more constant, more even and steeper than in the carnivorous cat and dog. We have suggested in a previous paper that it may be that the herbivora, with their rough bulky food must maintain a better gradient in their digestive tracts than is needed by the carnivora with their more concentrated, easily liquefied food and their short powerfully-muscled intestines.

Another point of interest is the rise in the gradient at the lower end of the cat's colon. Similar rises were observed in a number of the dogs. They were not observed in the rabbit, guinea pig and rat. It is suggestive that the first two animals defecate at comparatively rare intervals much as man does. They can thus comport themselves properly as pets in houses and they often seem to be quite constipated. The other animals, with the steep gradient down to the anus, defecate at short intervals, apparently as often as feces reach the rectum. A number of writers have claimed that in man there is a rise of tone up to the sigmoid or rectum (10), and one of us suggested several years ago that an increase in the steepness of the gradient up to the anus might be one of the principal factors in producing constipation (11). So far we have been able to get fresh segments from but one human colon. The cadaver was that of a man who died from a wide-spread terminal infection following a jejunostomy for an inoperable carcinoma of the stomach. The figures were as follows:

Cecum	*Colon*			*Rectum*
1	2	3	4	5
19.3	31.3	30.6	22.0	22.3

One cannot draw many conclusions from one case and a diseased one at that. There is, however, a definite downward gradient from the ascending colon to the rectum with perhaps a slight rise up to the anus. The low figure for the cecum, if normal for man, would account for the retention of fecal material in that region.

SUMMARY

Previous work on the small intestine and stomach showed gradients of catalase content and CO_2 production which the writers believe underlie and give rise to the gradients of rhythmicity, irritability and latent period which determine the direction of peristalsis.

Similar gradients are now shown to exist in the colon. The gradient of catalase content is steeper and more regular in the herbivorous rabbit and guinea pig than in the carnivorous cat and dog. The omnivorous rat has a fairly even gradient which is steep at the anal end. It is suggested that the herbivora with their rough bulky food and their thin-walled bowels need a more even gradient than the carnivora do.

In the cat and dog the lower end of the gradient is often reversed, that is, uphill towards the rectum and anus. It is suggested that this reversal saves these animals from frequent calls to defecation. The rabbit, guinea pig and rat defecate at short intervals, probably whenever material reaches the anus; the cat and dog empty their bowels more as man does and they sometimes are constipated. It is suggested that constipation may be due to an accentuation of the reversed gradient up to the anus. It is also suggested that the uneven gradient in the colon is associated with the well-known reverse peristalsis in that part of the gut. The more sluggish metabolism of the colonic muscle as compared with that of the small intestine was to be expected. No peculiarity could be observed in the catalase content of the ileocecal sphincter.

In one human case studied the gradient was quite similar to that in the dog, with the exception that the cecal figure was very low.

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BLOOD VOLUME

A Method for its Determination with Data for Dogs, Cats and Rabbits

WALTER J. MEEK and HERBERT S. GASSER

From the Physiological Laboratories of the University of Wisconsin and the Washington University Medical School

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Few problems in all physiology have been attacked more persistently or more ingeniously than that of determining the blood volume in mammals. The importance of such information from a theoretical as well as a practical standpoint has always been obvious. Although a score of methods have been described either for directly determining or indirectly calculating blood volume, we found that for work we wished to do on shock none completely met our needs. For example, very few of the methods allow a second determination to be made during the course of a single experiment. The final estimation of volume has usually depended on color comparisons, blood counts or other procedures in which certain errors if not absolutely necessary are, at least, easily made. It seemed to us that some method chemically accurate, gravimetric, if possible, might be of use if it could be devised.

Complete reviews of the blood volume literature may be found in the papers of Plesch (1), Behring (2), Dreyer and Ray (3) and Keith, Rowntree and Geraghty (4). It is here necessary only to comment on the various methods that have been employed.

The direct method of determining blood volume, that of bleeding and washing out the circulatory system, was introduced by Welcker (5) and used by many early investigators. With modifications it has been employed in recent years by Dreyer and his colleagues (3), (6), (7), Boycott (8), Rasmussen and Rasmussen (9) and others. Two possible errors have usually been recognized in the direct methods, namely: that pigment particularly from muscle might be included as hemoglobin and that some blood might not be washed out from the

spleen, bone marrow or parts of the portal system. The final calculations must be made in all cases by comparing the washings with an original sample of blood in regard to hemoglobin, number of red blood cells or content of solids. Any error made in these comparisons of dilute solutions becomes magnified when the original volume is calculated. Although these criticisms may be made, the direct method as used by Dreyer and Ray is probably the most accurate means of determining blood volume yet devised. It is, of course, limited in application since the life of the animal is sacrificed.

Indirect methods have been more generally used. These all depend on the addition of some substance to the blood stream and a subsequent determination of the dilution or concentration undergone either by the blood or the substance itself. Thus Vierordt (10), who seems first to have used such a technique, bled an animal, waited until he believed the tissue juices had restored the lost fluid and then determined the dilution of the red blood cells. Assuming that the blood received from the tissues an amount exactly equal to the volume withdrawn before any erythrocytes were regenerated, the blood volume could, of course, be estimated. The method, however, rested on two assumptions and the exact facts regarding these are not even yet completely known. Much the same comments may be made on Vierordt's method depending on ventricular output and circulation time.

Valentin (11) computed volume from determinations of solids before and after an injection of a measured amount of water. His values were too high since either salts were drawn in from the tissues or a portion of the injected water left the blood stream. Malassez (12) injected serum or whole blood with a known red blood cell count into an animal and from the resulting variation in the number of red cells made his computations. This technique was ingenious but hemolysis often introduced a large error. Cohnstein and Zuntz (13), improving on Valentin's method, diluted the circulation with isotonic NaCl of a known quantity and from the red blood cell dilution determined the blood volume. Kottmann (14) followed much the same procedures but determined the red cells by means of the hematocrit. Oerum (15) also used the same general method but made his determinations from comparisons of the hemoglobin content of the diluted blood with that of the original. Changes in the specific gravity of the blood, brought about by injections of known amounts of NaCl, were used by Sherrington and Copeman (16) to determine blood volume. The chief difficulties of all these methods lie not only in the technical errors incident

to blood counts, hematocrit and hemoglobin determinations but more especially in the extreme rapidity with which injected water and salt solutions leave the blood serum.

Two injection methods may be mentioned which differ from the preceding in that the substances injected do not leave the blood stream rapidly. Behring (2) investigated blood volume by studying the dilution undergone by a measured amount of tetanus antitoxin when injected into the blood. This method he recommended clinically.

A method of excellent promise has recently been described by Keith, Rowntree and Geraghty (4). It consists of an injection of a known amount of the dye, vital red, and a determination of its dilution in the blood. The dye is harmless in the doses employed and repeated determinations may be made. Although of great service clinically, in animal experiments the method often fails on account of slight hemolysis or lipaemia. In addition the dye cannot now be generally obtained.

Grehant and Quinaud (17) in 1883 introduced the CO inhalation method of determining blood volume. This consisted in breathing a known amount of CO and then determining the degree of saturation undergone by the hemoglobin of the blood. The CO capacity of the blood having previously been determined, the total volume could easily be calculated. Haldane and Smith (18) improved the method and later it was still further refined by Zuntz and Plesch (19) and Boycott and Douglas (20). The CO inhalation method has never given very constant results and, although it has been of some value clinically, it can scarcely be said to have a great degree of accuracy.

DETERMINATION OF BLOOD VOLUME WITH ACACIA

In casting about for some inert substance that could be injected into the circulation and then recovered, the conditions necessary for an indirect blood volume determination, the attention of one of us, Doctor Gasser, was directed to gum acacia. It seemed probable that this substance even in large doses might have no effect on any vital function and that it might be recovered from the blood as furfurol-phloroglucid by the method of Kröber (21). On experiment we found that both of these possibilities were true. Acacia may be injected into a dog intravenously until the animal's blood becomes a 7 per cent or 8 per cent solution without obvious changes in the respiratory or nervous systems, or in the circulatory, at least so far as can be told from general blood

pressure. Under a local anaesthetic acacia may be injected into an intact animal until the blood is a 4 per cent solution without the general condition of the animal being in any way affected. After such injections acacia may be recovered as phloroglucid from a blood sample.

As a general plan for our method we proposed the following procedures: First, to determine the amount of acacia that could be recovered after adding a known amount to a 10 cc. sample of blood. This we shall speak of as the "control." Second, to inject such an amount of acacia into the animal that, on the assumption of his having a certain percentage of his body weight as blood, each 10 cc. of his blood would have exactly the same amount of acacia as the control. Third, allow ample time for the injected acacia to mix with the blood. Fourth, after time for mixing draw a 10 cc. sample of blood and determine its acacia content. This will be spoken of as the "blood sample." The assumed blood volume in percentage of body weight would then be to the true blood volume in percentage of body weight as the acacia content of the blood sample is to the acacia content of the control.

Acacia has in all our work been determined as furfurol-phloroglucid by the method of Kröber (21). We have made no important modification of his technique. It consists in distilling the acacia or blood containing acacia in the form of furfuraldehyde from HCl solution of specific gravity 1.06. To this distillate is added 1 per cent phloroglucin dissolved in HCl of specific gravity 1.06. The distillate should stand 24 hours to insure a complete precipitation of the phloroglucid. The precipitate is then caught on a Gooch crucible, dried four hours at 100°C., cooled in a desiccator and weighed. One hundred milligrams of acacia on the average yield about 26 mgm. of phloroglucid. After passing the crucible the filtrate is greenish in color showing that some of the phloroglucid is in solution. For 550 cc. of distillate Kröber estimates this amount to be 5.2 mgm. For the control and blood sample we must disregard this correction since, as shown later, the amount of phloroglucid recovered from these samples is in itself a constant fraction of the total amount of acacia added. To be sure that no more furfurol is coming over we have often let a drop of the distillate fall on a paper moistened with aniline acetate. A pink ring indicates the presence of furfuraldehyde. All determinations which had to do with establishing the method were done in duplicate. The method is in part empirical but it is accurate if the amount of phloroglucin added for precipitation is from 1.25 to 3 times the yield in phloroglucid. We have accordingly added from 5 to 10 cc. to our distillates.

In actual experiments we have found it most convenient to use acacia solutions of the following strengths. For our control we have added 10 cc. of 1 per cent acacia to 10 cc. of freshly drawn blood. The hydrochloric acid was not added until 10 minutes later. As an injection, we have generally given 4 cc. of a 20 per cent acacia solution for each kilo of body weight. The acacia for injection is made up in a 0.9 per cent NaCl solution. In some of our earlier experiments the acacia was dissolved in water. The presence or absence of the NaCl has seemed to make no difference in the determinations. If the animal should have 8.4 per cent of his body weight in blood then the amount injected would add exactly 10 mgm. of acacia to each cubic centimeter of his blood, an amount exactly equal to what has been added to the control. In all calculations the specific gravity of blood has been figured as 1.050. After the acacia determinations are made the blood volume may be calculated from the following proportions:

$$\frac{8.4 \text{ per cent}}{\text{Blood volume in per cent of body weight}} = \frac{\text{Weight of phloroglucid in blood sample}}{\text{Weight of phloroglucid in control}}$$

Or if the total volume is desired at once in cubic centimeters the proportion may be stated in this way:

$$\frac{20 \times \text{cc. of acacia injected}}{\text{Blood volume in cc.}} = \frac{\text{Weight of phloroglucid in blood sample}}{\text{Weight of phloroglucid in control}}$$

This volume must be corrected for the difference between the amount of blood drawn and the acacia injected.

In a number of experiments we determined the yield of the acacia itself in phloroglucid. From this we knew the total amount injected. Dividing this amount by the amount recovered in the blood sample gave us another means of determining blood volume. This formula was used to check up the other determinations.

Conditions which the method must satisfy. There are certain criticisms which the acacia method in common with all other indirect methods of determining blood pressure must meet. The most important of these are:

1. Can the acacia be recovered quantitatively from the blood?

2. Does any acacia leave the blood stream during the period allowed for mixing?

3. In the same period does the injected acacia in any way expand the blood volume?

In regard to the recovery of acacia from blood it was soon found that the total amount added could not be regained. To illustrate, if 10 cc. of 1 per cent was found to yield 26 mgm. of phloroglucid, the same amount in blood would produce only about 21 mgm. The amount lost varied with the kind of blood and with its quantity. More could be recovered from rabbits' blood than from dogs'. The percentage of acacia regained from varying amounts of blood may be seen from the following figures which are averages from a number of experiments.

10 cc. of 1 per cent acacia in 5 cc. blood yielded 90 per cent of total
10 cc. of 1 per cent acacia in 10 cc. blood yielded 83 per cent of total
10 cc. of 1 per cent acacia in 20 cc. blood yielded 76 per cent of total

We are unable to say with any certainty what becomes of this acacia. It is quite possible, however, that some of the aldehyde formed in the distillation may combine with substances in the blood and so fail to pass over. Pentoses are also known to form humin compounds with some of the amino acids.

At first sight it would seem that this loss of acacia, which is roughly proportional to the quantity of blood, would render our method impossible. To estimate the loss would require a knowledge of the blood volume, the very thing that the method is designed to discover. In actual practice, however, it is not the amount of blood which is the variable but the amount of acacia which a given quantity of blood receives on injection. If the blood volume should by chance be exactly equal to our assumed 8.4 per cent, then each 10 cc. of blood would receive an amount exactly equal to that in the control. Should the blood volume be either twice or one-half that expected, then each 10 cc. of blood would receive either one-half or twice that in the control. These figures represent the widest possible limits that might be expected for variations in blood volume. The important question would then seem to be whether on adding such amounts of acacia to 10 cc. samples of blood we can recover all or any constant fraction of the total. On taking 10 cc. quantities of blood and adding acacia to them in such amounts as the blood would receive in the body should it vary from twice to one-half normal, the following data were secured.

TABLE I

Showing the amount of acacia recovered as phloroglucid when 1 per cent acacia in varying quantities is added to 10 cc. samples of blood

EXPERIMENT NUMBER	5 CC. OF 1 PER CENT ACACIA IN 10 CC. BLOOD	10 CC. OF 1 PER CENT ACACIA IN 10 CC. BLOOD	20 CC. OF 1 PER CENT ACACIA IN 10 CC. BLOOD	30 CC. OF 1 PER CENT ACACIA IN 10 CC. BLOOD
	mgm.	*mgm.*	*mgm.*	*mgm.*
1	11.9	24.0	47.9	
2	12.2	24.6	50.9	
3	11.7	23.1	46.0	79.0
4	10.1		40.5	
5	12.3	25.3	54.6	
6	11.6	24.7	46.7	
7	11.8	23.2	50.6	78.0

These figures show clearly that almost exactly the same proportion of acacia may be recovered as phloroglucid from 10 cc. of blood no matter whether 5, 10 or 20 cc. of 1 per cent acacia have been added. Although 10 cc. of blood combine with more acacia when 20 cc. of the 1 per cent solution are added, the percentage yield remains the same for all three concentrations. The actual recovery in per cent may easily be determined in any of the experiments in which a preliminary analysis of the acacia alone was made. In experiment 1, 5 cc. of the 1 per cent acacia were analyzed and found to yield 15.8 mgm. of phloroglucid, including here, of course, the correction for the amount left in solution in the filtrate. The amounts recovered from 5, 10 and 20 cc. in blood were therefore 75.3 per cent, 75.9 per cent and 75.7 per cent. In experiment 2, 5 cc. of 1 per cent acacia alone yielded 15.55 mgm., the recovery for the three samples therefore being 78.4 per cent, 79.1 per cent and 81.8 per cent. All the experiments are not quite so satisfactory as these two but they all seem sufficiently well in agreement to justify our contention that the phloroglucid recovered from our control may be compared with that from the blood sample to determine volume even though the acacia in the two may vary in concentration. Most of the variations found in table 1 are really within the limits allowed for gravimetric methods. Variations of a few per cent here are fortunately almost without any influence on the final determinations of blood volume. In experiment 2, for example, there is a difference of 3.4 per cent between the percentage recovery of phloroglucid in the 5 and 20 cc. samples. It would take a difference of about 10 per cent, however, to make 0.5 per cent difference in the final determination of blood volume.

It cannot be denied that the conclusions just reached seem inconsistent with the evidence already submitted that with increasing amounts of blood we get decreasing yields of acacia as phloroglucid. This would seem to indicate that the blood contained some substance with which the acacia or one of its decomposition products united according to the general law of chemical proportions. If this were true on increasing the amount of acacia added to 10 cc. samples of blood we should expect to get a steady increase in the amount of acacia recovered as phloroglucid. On the contrary, we have found that within limits the same percentage of acacia is always recovered as phloroglucid from a given amount of blood. There is, however, a possible explanation for such a contradiction. As the concentration of the acacia in the 10 cc. samples increases it might form polymers with some unknown constituent of the blood instead of combining according to the law of chemical proportions. There are several facts that indicate such a possibility. Pentosans are known to polymerize in the formation of humin compounds. Furfurol has been shown to combine with phloroglucin in varying proportions according to the concentrations of each. There must, of course, some time be an end to the power of polymerizing and we should accordingly find a concentration of acacia that would give more than the expected yield. Twenty cubic centimeters of 1 per cent acacia in 10 cc. of blood is probably very near this limit. The yield for 20 cc. as shown by table 1 is, in most of the experiments, very close to four times that for 5 cc. but in several it is high and the average is somewhat above what is expected. When 30 cc. of 1 per cent acacia are added the limit in which we recover our expected amount is definitely passed and instead of the yield being 70.2 mgm. it is 79 mgm. Whatever the explanation may be the important thing for our method is the undoubted fact that we can regain exactly the same percentage of acacia as phloroglucid from a 10 cc. blood sample if it has anywhere from 5 to 20 cc. of 1 per cent acacia in it. These limits represent the concentrations found in blood volume determinations of one-half to two times normal.

The second condition that our method must satisfy is that no acacia shall be lost during the period allowed for mixing with the blood. It is of course, well known that many substances begin to leave the blood stream as soon as injected. Although this might not be expected of a compound having as large a molecule as acacia, it is a matter that must be determined as carefully as possible.

We have usually allowed ten minutes after injection for the acacia

to mix thoroughly with the blood. This time has seemed longer than really necessary but we have preferred to be on the safe side. Keith, Rowntree and Geraghty (4) allowed three minutes for vital red to mix with the blood. Plesch (1) considered three to four minutes ample for mixing after injection of sodium chloride. Sherrington and Copeman (16) found mixing complete in the rabbit after as short a period as thirty to forty seconds. Acacia in a 20 per cent solution is a rather viscous fluid and could not be expected to mix as quickly as NaCl. Granting fifteen seconds as the circulation time, ten minutes would allow the acacia to travel through the body forty times, certainly opportunity for mingling completely with the blood. Our analyses not only justify such a conclusion but show that any time after five minutes the mixing is complete. The following table compares analyses of blood samples taken five and ten minutes after the injection of acacia was completed.

TABLE 2

Showing the amounts of acacia recovered as phloroglucid from samples taken at five and ten minute intervals after injection

EXPERIMENT NUMBER	FIVE MINUTE SAMPLE	TEN MINUTE SAMPLE
	mgm.	*mgm.*
1	20.0	20.6
2	20.9	20.6
3	23.5	24.1
4	20.8	20.0
5	17.5	17.5
6	20.4	18.6
7	17.6	17.6
8	22.5	22.9
9	19.4	19.7

With the exception of experiment 6, for which we have no explanation, these analyses show a very close agreement between the five and ten minute samples. Three important points are made clear by these data; first, that the acacia does mix thoroughly with the blood; second, that we have allowed ample time for mixing; and third, that during this period there is no loss of acacia from the blood stream.

Ultimately the acacia, of course, disappears from the blood. We have not attempted to work out the exact curve of its disappearance but a number of interesting observations have been made which may have some bearing on the use of acacia as an injection fluid. In one

experiment after injection we found the following amounts present in 10 cc. samples at various times up to five hours.

	mgm.
3 minutes	15.9
12 minutes	15.8
18 minutes	15.1
60 minutes	14.4
5 hours	12.2

In this case the decrease at the end of the first hour was about 8.8 per cent and this increased to 22.8 per cent at the end of five hours. In another experiment at the end of twenty-four hours there was still present 50 per cent of the original amount injected. In two experiments in which perfectly normal dogs received intravenous injections of acacia in large doses, 12 to 15 cc. per kilo of a 20 per cent solution, there was found in the blood forty-eight hours later 42 per cent in one and 33 per cent in the other.

The third criticism which our method has to meet is that the acacia must not of itself expand the blood volume. This might be brought about mechanically by the injection of large quantities or by the osmotic properties of the acacia. The first of these is easily controlled. In large animals where the amount to be injected exceeds the blood drawn for the control we bleed an amount equal to the quantity to be injected. It is, of course, well known that after hemorrhages fluid is drawn in from the tissues to restore the depleted blood and that this process begins immediately. Sherrington and Copeman (16) found after drawing 32 cc. of blood from a six-pound rabbit that the maximum reduction in specific gravity was not reached until fifteen to twenty-five minutes afterward. From this it does not seem that drawing an amount of blood equal to the volume injected could introduce an appreciable error for our injection begins at once after the bleeding and it is finished usually within a few minutes. In small animals the amount drawn for controls may be greater than the amount of acacia to be injected. In these cases it is often convenient to inject 8 cc. per kilo of 10 per cent acacia instead of 4 cc. per kilo of 20 per cent.

That the osmotic pressure of the acacia has not influenced the blood volume during the mixing period is shown by the data in table 2. It is evident that there would be a constant decrease in the amounts recovered in the ten minute samples, if the blood volume were being expanded, unless the total expansion took place within the first five-minute period. Evidence obtained by following the hemoglobin

shows, however, that this is not the case. This conclusion is also borne out by numerous blood counts. At the end of the mixing period there has never been a decrease in the red blood count that was not almost entirely accounted for by the direct dilution resulting from the injection. In some cases there has been a slight increase after injection, the meaning of which is not clear. In table 3 are given the data covering these points.

TABLE

RED BLOOD CELL COUNT BEFORE INJECTION	ESTIMATED DILUTION OF THE BLOOD DUE TO THE VOLUME OF ACACIA INJECTED	RED BLOOD CELL COUNT 10 MINUTES AFTER INJECTION.	DECREASE IN RED BLOOD CELLS.
	per cent		*per cent*
8,320,000	10	7,520,000	9
8,640,000	16	8,800,000	Increase
4,650,000	5	4,792,000	Increase
4,368,000	9	4,032,000	7
5,800,000	12	5,008,000	13
7,040,000	15	6,236,000	11
8,440,000	9	7,600,000	9
6,583,000	13	5,405,000	17
6,700,000	19	5,840,000	12
8,750,000	4	8,500,000	3

Even after the mixing period no further dilution appears in the blood at least for a considerable period of time. Data from two experiments covering this point are here given.

December 2. Dog

10.10 a.m.	Blood count equals	6,016,000
10.34 a.m.	Acacia injected	
10.40 a.m.	Blood count equals	5,608,000
10.59 a.m.	Blood count equals	5,920,000
11.40 a.m.	Blood count equals	5,536,000

December 12. Dog

9.45 a.m.	Blood count equals	6,600,000
10.00 a.m.	Acacia injected	
10.37 a.m.	Blood count equals	6,400,000

A 20 per cent solution of acacia in a 0.9 per cent NaCl has, however, an osmotic pressure appreciably higher than that of the blood serum and it would seem that sooner or later this influence would exert itself by drawing in water from the tissues. In certain experiments we have

evidence that in the course of an hour or two this really may take place. Our injection of 4 cc. per kilo of 20 per cent acacia converts the plasma of a dog into a 1.38 per cent acacia solution on the basis of the plasma being 60 per cent of the blood and the total blood volume being about 9 per cent of the body weight. Two things would, of course, happen to the plasma in this case. Its osmotic pressure would be lowered by the dilution undergone on adding the acacia, but this would be more than compensated for by the increase due to the higher osmotic pressure of the acacia. The dilution would be about 1/13.5 (7.4 per cent) of the plasma volume. This would lower the colloidal osmotic pressure of the plasma about 2.70 mm. Hg. using Bayliss' (22) figures for the osmotic pressure of serum. Bayliss found 7 per cent sodium acacia to have an osmotic pressure of 39 mm. Hg. If calcium acacia is the same, a 1.38 per cent solution would have a pressure of 7.7 mm. Hg. The plasma would thus have its colloidal pressure lowered 2.70 mm. and raised 7.7 mm. bringing it to 45 mm. Hg. This is an increase of 12.5 per cent above the original. We might then expect a plasma dilution of about the same percentage due to the inflow of tissue juices. But the plasma is only about 60 per cent of the total blood volume; therefore the total blood volume could be expanded only about 7.5 per cent. It should be recalled at this point that we have already submitted data showing that at the end of an hour something like 9 to 10 per cent of the acacia had left the blood stream. Data for only one experiment have been given but the results are in harmony with those of several others. This decrease in acacia is due only in part to expansion of the blood, since the maximum dilution as determined by the hemoglobin content of the blood has never been found to be greater than 7.5 per cent, an amount which corresponds closely to the calculated theoretical expansion. Data covering this point may be seen in the following table.

It will be noticed from table 4 that the maximum expansion occurs before the end of an hour. The 9 to 10 per cent decrease in the acacia of the blood stream is, therefore, in part a real loss and additional evidence of this fact is afforded by a reaction for pentose which may be obtained from the urine at this time. From the foregoing data and discussion it is evident that the osmotic properties of the acacia are not a seriously disturbing element in the determination of blood volume.

Second determinations of blood volume in the same animal. To be most useful a blood volume method should be capable of being used at frequent intervals on the same animal. The acacia method may be

TABLE 4

Increase in volume of blood as determined by the hemoglobin content at various periods after injection

EXPERIMENT NUMBER	MINUTES AFTER INJECTION	INCREASE IN BLOOD VOLUME
		per cent
47	10	5.6
	30	6.6
	60	6.0
48	30	3.1
49	39	−2.8
50	6	7.3
	25	7.5
51	27	4.0

used at least twice successfully in the same experiment. For the second control 10 cc. of blood are drawn and to it are added 5 cc. of the 1 per cent acacia solution. An injection equal to one-half the first is now made, the usual time for mixing allowed and then the second blood sample drawn. The acacia content of these samples as phloroglucid is then determined. In order to make use of these data the amount of acacia remaining in the blood from the first injection must be known. This is easily found by subtracting one-half of the first control from the second. If this difference is now subtracted from the second blood sample, the equation may be completed as follows:

$$\frac{\text{8.4 per cent}}{\text{Blood volume in per cent of body weight}} = \frac{\text{Pg. in second blood sample} - (\text{Pg. in second control} - \tfrac{1}{2}\text{ first control})}{\tfrac{1}{2}\text{ First control}}$$

Immediately succeeding determinations will be found to correspond if the analyses are done in duplicate and the duplicates check each other closely. The necessity of perfection in technique is apparent when one considers that an error of only 0.1 mgm. may change the second blood volume as much as 0.1 per cent of body weight. This is due to the small weights compared in the ratio used for the second determination. In table 5 are given the results of three such determinations.

In tables 6, 7, 8, may be found the data from a number of blood volume determinations on dogs, cats and rabbits. We have made no

particular effort to compare these results with those of previous workers. Plesch found the average blood volume in nine healthy dogs was

TABLE 5

EXPERIMENT	FIRST CONTROL	FIRST BLOOD SAMPLE	SECOND CONTROL	SECOND BLOOD	FIRST BLOOD VOLUME	SECOND BLOOD VOLUME
	mgm.	*mgm.*	*mgm.*	*mgm.*	*per cent*	*per cent*
20	23.3	23.5	33.3	33.3	8.7	8.8
21	24.1	23.2	35.7	34.5	9.1	9.7
22	23.9	20.9	33.0	32.0	10.0	9.6

TABLE 6

Blood volume of dogs in percentage of body weight

WEIGHT IN	PHLORO-GLUCID IN CONTROL	PHLORO-GLUCID IN BLOOD SAMPLE	CORRECTION FOR BLEEDING AND INJECTING	BLOOD VOLUME IN PER CENT OF BODY WEIGHT
kgm.	*mgm.*	*mgm.*	*cc.*	*per cent*
8.80	24.1	21.2	−15.2	9.36
8.00	25.0	19.5	− 8.0	10.65
7.56	23.9	22.6	− 5.2	8.80
4.60	20.0	18.8	−12.8	8.60
9.51	18.5	15.6	−13.0	9.81
5.10	19.4	19.7	+ 1.6	8.28
8.40	22.6	20.8	0.0*	9.56
5.02	21.2	15.7	+ 2.0	11.38
4.84	27.6	21.1	+ 5.7	11.02
5.40	21.1	18.4	− 1.6	9.59
7.80	23.3	23.5	0.0*	8.72
5.42	22.6	23.1	+53.4	9.24
8.28	23.8	20.4	− 7.1	9.69
12.06	28.8	23.1	−28.3	10.15
11.20	20.1	15.8	−29.8	10.32
13.20	23.1	18.6	0.0	10.43
9.40	20.9	17.2	0.0	10.20
20.00	24.0	22.3	0.0*	9.47
Average				9.74

*In these experiments 12 cc. per kilo of 7 per cent acacia were used; 8.4 of the formula was therefore replaced by 8.8.

8.57 per cent of body weight with variations from 7.42 per cent to 10.46 per cent. For rabbits, Dreyer and Ray give the blood volume as 4.92 per cent of body weight although it should be noted that they

do not believe the ratio of blood volume to body weight is a constant in normal animals of any given species. Our results are therefore somewhat higher than any previously reported.

TABLE 7

Blood volume of cats in percentage of body weight

WEIGHT IN	PHLORO-GLUCID IN CONTROL	PHLORO-GLUCID IN BLOOD SAMPLE	CORRECTION FOR BLEEDING AND INJECTING	BLOOD VOLUME IN PER CENT OF BODY WEIGHT
kgm.	*mgm.*	*mgm.*	*cc.*	*per cent*
3.44	22.2	37.8	+13.3	5.33
2.75	20.7	27.9	+14.0	6.76
3.90	20.9	31.7	+ 7.4	5.72
3.50	23.6	41.0	+ 6.0	5.00
2.72	21.0	35.0	0.0	5.04
1.50	21.6	30.8	+ 5.0	6.23
2.20	23.1	42.8	0.0	4.53
4.30	16.0	26.0	0.0	5.41
Average				5.50

Note: 7 per cent acacia was used in the last experiment of the table.

TABLE 8

Blood volume of rabbits in percentage of body weight

WEIGHT IN	PHLORO-GLUCID IN CONTROL	PHLORO-GLUCID IN BLOOD SAMPLE	CORRECTION FOR BLEEDING AND INJECTING	BLOOD VOLUME IN PER CENT OF BODY WEIGHT
kgm.	*mgm.*	*mgm.*	*cc.*	*per cent*
2.00	21.2	36.5	+6.0	5.18
3.00	18.3	34.9	−2.0	4.32
2.50	24.6	34.3	+2.0	6.10
1.80	23.8	42.8	0.0	4.67
2.10	27.0	38.8	0.0	5.84
2.57	30.2	48.1	+6.4	5.42
2.17	34.2	45.0	+3.6	6.56
Average				5.44

SUMMARY

A method of blood volume determination by means of an acacia injection has been reported. The method depends on an injection of a known amount of acacia into the blood stream and a determination of the dilution undergone by the acacia after time for mixing with the

blood has been allowed. The acacia has been determined as furfurol-phloroglucid by the method of Körber. The injection of 4 cc. of 20 per cent acacia per kilo has been shown to be harmless to the animal, neither does it expand the blood volume during the time necessary for a determination.

By this method the average blood volume of the dog has been found to average 9.72 per cent of the body weight. For the cat and rabbit the percentages are 5.50 and 5.44 respectively.

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QUANTITATIVE STUDIES ON INTRACELLULAR RESPIRATION

IV. The Rates of CO_2 Production by Starved and Fed Paramecia, and their Possible Relation to the Rates of Oxidation in the Unfertilized and Fertilized Sea Urchins Egg

E. J. LUND

From the Department of Animal Biology, University of Minnesota

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The purpose of the present paper is *a*, to present data which show that the carbon dioxide production by Paramecium is increased very markedly after ingestion of food such as yeast or egg yolk, thus confirming the results presented in the previous paper on the relation of state of nutrition and rate of oxygen consumption; and *b*, to show that this marked increase in respiration is not necessarily accompanied by cell division, thus being closely parallel to the condition found by others in the egg of the sea urchin.

For determining such very small quantities of carbon dioxide as are produced by 1 or 2 cc. of a suspension of Paramecium, a method was employed, the description of which has been given elsewhere together with tests of its accuracy (1).

The conclusions from results of the experiments reported below, using this method, do not depend for their validity upon the question as to whether the method is capable of measuring the absolute quantity of carbon dioxide produced by the organisms, for the measurements of the carbon dioxide produced by the fed and starved Paramecia are primarily comparative, which is all that is required for establishing the conclusions. As a matter of fact, the results very probably also represent the absolute quantities of CO_2 produced by the Paramecia during the periods of the experiments, within limits of error of the method when such small amounts of carbon dioxide are measured. When the method is properly carried out, the error in measuring amounts of carbon dioxide equal to that liberated from 1 mgm. of Na_2CO_3 by an acid is about equal to 5 per cent.

The results of four experiments are given in the following four tables. The procedure was practically the same in all of them. They differed only in the degree of starvation before the experiments were begun and in the number of Paramecia tested in each experiment. The description of the plan and procedure of the experiment, table 1, will apply to all the others except for minor differences which are noted in the descrip-

TABLE 1

Paramecia were starved 24 hours before being used. The protoplasm was clear. Figures refer to the number of cubic centimeters n/100.3 HCl *necessary for neutralization of the free* $Ba(OH)_2$ *in each bottle. Twenty cubic centimeters* $Ba(OH)_2$ *were added to each bottle. Duration of experiment, 23 hours. Temperature 26°C.*

BOTTLE	TITRATED AT ONCE	TITRATED AT END OF 23 HOURS			
	Controls. No yeast added			Yeast added	
	1	2	3	4	5
	Blanks	2 cc. Paramecia + 3 drops tap water. Starved	2 cc. "native tap" + 3 drops tap water. No Paramecia	2 cc. Paramecia + 3 drops yeast. Fed	2 cc. "native tap" + 3 drops yeast. No Paramecia
1	21.40	19.60	20.15	18.80	20.15
2	21.35	19.55	20.30	18.80	20.20
3	21.40	19.57			20.12
Average.	21.38	19.57	20.22	18.80	20.15

	cc.
Average cc. n/100.3 HCl equivalent of CO_2 absorbed from air in bottle and 2 cc. "native tap." Difference between columns 1 and 3	1.16
Average cc. n/100.3 HCl equivalent of CO_2 produced by dead yeast. Difference between averages, columns 3 and 5	0.07
Average cc. n/100.3 HCl equivalent of CO_2 produced by starved Paramecia. Difference between averages, columns 2 and 3	0.65
Average cc. n/100.3 HCl equivalent of CO_2 produced by fed Paramecia. Difference between averages, columns 4 and 5	1.35

tion above each of the tables. Paramecia from a pure line were transferred gradually from native hay infusion to tap water and starved for various periods of time. This suspension was concentrated with the centrifuge just before the beginning of the experiment. The words "native tap" in columns 3 and 5 of the tables mean the tap water medium into which the Paramecia had finally been transferred after washing. Fresh compressed yeast suspended in fifty times its

volume of distilled water was killed by boiling for one minute, quickly cooled and then washed in tap water by means of the centrifuge. This suspension of yeast was used as food for the animals. The figures in table 1, column 1, are the number of cubic centimeters n/100.3 HCl which were necessary for neutralization of 20 cc. of the stock solution of Ba $(OH)_2$, i.e., before absorption of any CO_2. The procedure in adding the $Ba(OH)_2$ solution to the bottles and titration has been given in the description of the method.

The numbers in columns 2, 3, 4 and 5 give the number of cubic centimeters n/100.3 HCl necessary to neutralize the remaining free base after absorption of all CO_2 liberated from various sources, as indicated in the separate columns, during the period of the experiment which was in this case twenty-three hours. The average CO_2 produced by the unfed Paramecia in 2 cc. of the concentrated Paramecium suspension in tap water during twenty-three hours is equal to the difference between the averages in columns 2 and 3. The average in column 3 is a control to determine how much free $Ba(OH)_2$ in terms of cubic centimeters n/100.3 HCl remained after all free CO_2 from the air in the bottles and the dissolved CO_2 in the 2 cc. tap water medium in which the Paramecia lived (designated "native tap" in the tables), had been absorbed.

The bottles in column 2 were set up exactly like those in column 3 except that Paramecia were present. It is therefore obvious that the numbers in column 2 represent the amount of free $Ba(OH)_2$ in terms of cubic centimeters n/100.3 HCl which remained after all the free CO_2 present in the air in the bottle, plus that amount which was dissolved in the 2 cc. tap water medium introduced with the Paramecia, *plus the amount of CO_2 produced by the Paramecia in 2 cc. of the stock suspension, during 23 hours,* had been absorbed. The figures in column 2 are smaller than those in column 3, thus showing that more $Ba(OH)_2$ had been neutralized in these bottles than in those of column 3, i.e., more CO_2 had been absorbed in bottles of column 2 than in those in column 3. This difference in cubic centimeters n/100.3 HCl is therefore equivalent to the amount of CO_2 produced by the unfed Paramecia in 2 cc. of the Paramecium suspension during 23 hours. In this experiment the CO_2 produced by the starved animals was equivalent to 0.65 cc. n/100.3 HCl.

The bottles in columns 4 and 5 are *exact duplicates* respectively of the bottles in columns 2 and 3, except that food (three drops of the suspension of yeast cells in tap water) was added. The figures in

column 5 therefore give the amount of free $Ba(OH)_2$ remaining after all CO_2 present in the air in the bottles, plus the CO_2 dissolved in the 2 cc. tap water medium, plus any CO_2 produced by the killed yeast cells, had been absorbed. That the killed yeast did not produce any CO_2 is shown by the fact that the average amount of $Ba(OH)_2$ left in the bottles in columns 3 and 5 is the same within the limits of error in titration. The average amount of CO_2 produced by the fed Paramecia, column 4, is therefore equivalent to the difference between 20.15 cc. n/100.3 HCl, column 5, and 18.80 cc. n/100.3 HCl, column 4, or 1.35 cc. n/100.3 HCl. The quantity of CO_2 produced by the starved Paramecia was therefore only one-half as much as that produced by the same number of fed Paramecia during the same period, all other conditions being identical.

Both the actual amounts of CO_2 produced by the Paramecia in terms of cubic centimeters n/100.3 HCl—0.65 cc. and 1.35 cc. respectively—and the difference in these same amounts, or 0.70 cc., are several times greater than any errors entering into the titrations or other sources of error in any part of the experiment. The maximum difference between any two of the duplicate titrations is 0.15 cc. n/100.3 HCl. Taking averages of the duplicate tests reduces this source of error to one-half or one-third. This degree of uniformity in the results completely justifies confidence in the method employed and confirms its reliability for determining such small amounts of CO_2 as are dealt with in these experiments on Paramecium and as was shown in tests of the method by using known amounts of CO_2 in the form of Na_2CO_3 (1).

Furthermore, the following tables show an equal degree of accuracy in the results. The number of Paramecia in each 2 cc. of suspension must be large enough so as to produce a sufficient amount of CO_2 for measurement. The smallest quantity of CO_2 which can be measured to within about 5 per cent error, is roughly equal to the amount of CO_2 set free from 1 mgm. of Na_2CO_3 by an acid, while for merely detection of differences this amount of CO_2 may be as small as the amount of CO_2 equivalent to 0.1 to 0.2 cc. n/100 HCl, or the amount of CO_2 set free from about 0.05 to 0.1 mgm. of Na_2CO_3 by an acid. This assumes of course that the technique of the experimenter in carrying out the experiment is satisfactory and as described. Fortunately in this method the accuracy of procedure is indicated by the degree of uniformity in the titrations of the free $Ba(OH)_2$ in the various duplicate bottles.

The experiment in table 2 is practically a repetition of the one in table 1 except that a different suspension of Paramecia was used; the duration of the experiment was 22 instead of 23 hours and the temperature was 24°C. instead of 26°C. The average amounts of CO_2 produced by equal numbers of starved and fed Paramecia were respectively equivalent to 0.44 cc. and 0.83 cc. n/100.3 HCl, hence the

TABLE 2

Paramecia starved 48 hours in tap water previous to the experiment. Protoplasm was transparent. Twenty cubic centimeters $Ba(OH)_2$ *were added to each bottle. Duration of experiment, 22 hours. Figures in the table represent the number of cubic centimeters n/100.3 HCl required to neutralize the free* $Ba(OH)_2$ *in each bottle. Temperature 24°C.*

BOTTLE	TITRATED AT ONCE	TITRATED AT END OF 22 HOURS			
		Controls. No yeast added		Yeast added	
	1	2	3	4	5
	Blanks	2 cc. Paramecia + 3 drops tap water. Starved	2 cc. "native tap" + 3 drops tap water. No Paramecium	2 cc. Paramecia + 3 drops yeast. Fed	2 cc. "native tap" + 3 drops yeast. No Paramecium. Control
1	21.00	19.60	20.05	19.15	19.90
2	20.95	19.50	19.98	19.12	19.95
3	20.90	19.60		19.05	19.95
Average.	20.95	19.57	20.01	19.11	19.94

	cc.
Average cc. n/100.3 HCl equivalent of CO_2 absorbed from air in the bottle and 2 cc. "native tap." Difference between columns 1 and 3	0.94
Average cc. n/100.3 HCl equivalent of CO_2 produced by dead yeast. Difference between averages in columns 3 and 5	0.07
Average cc. n/100.3 HCl equivalent of CO_2 produced by starved Paramecium. Difference between averages in columns 2 and 3	0.44
Average cc. n/100.3 HCl equivalent of CO_2 produced by fed Paramecium. Difference between averages in columns 4 and 5	0.83
Greatest difference in any duplicate titration	0.10

amount of CO_2 produced by the Paramecia fed with yeast was about twice as great as that produced by the same number of starved ones in the same length of time.

In order to determine whether the increased CO_2 production by fed Paramecia was specifically associated with and a result of cell division as such, or whether it was a result of ingestion, digestion and assimilation of the food by the cell leading to growth but *not cell divi-*

sion as such, the duration of the experiment was shortened to 8 and 9 hours so as to determine the CO_2 produced by the starved and fed Paramecia *before* any divisions of cells had taken place.

That cell division does not take place under the conditions of these experiments was shown in the previous paper on the relation of oxygen consumption to state of nutrition. In addition to the reasons given in the previous paper for believing that no cell division took place within eight to nine hours under the conditions of the experiments, it *was shown by direct counts of the number of Paramecia that the fed ones had not increased in number during this period.* From the results of the experiment in table 4 (really a double experiment, using yeast and yolk of hen's egg as food in two parts of the experiment) it is seen that the amount of CO_2 produced by the yeast-fed Paramecia was equivalent to 1.19 cc. n/100.3 HCl, while that of the starved ones was equivalent to 0.35 cc. n/100.3 HCl, or over three times as much CO_2 was produced by the yeast-fed Paramecia in 8 hours as by the same number of starved ones. Those fed with yolk produced two and one-half times as much CO_2 as the starved ones. In other words, both yolk and boiled yeast give the same result. Counts showed that no increase in the number of Paramecia during the 8 hours had occurred, hence it is clearly proved that cell division as such is not responsible for the observed increase of CO_2 production with ingestion of food by the cell.

The results of the experiment in table 3 are similar but owing to the small number of Paramecia in each 4 cc. of the Paramecium suspension used, the quantities of CO_2 produced by the Paramecia are too small for drawing definite conclusions. The table is added to show the degree of uniformity obtainable and to indicate the minimal quantities of CO_2 which can be dealt with by the method.

The above experiments on CO_2 production confirm in every respect those on the consumption of oxygen by fed and starving Paramecia when Winkler's method for oxygen determination was used, and will serve as a secure basis for interpretation of the relations of oxidations to other cell processes in Paramecium (2). It is interesting to note that the magnitude of the increase in rate of oxidations after feeding a starved Paramecium is similar, although somewhat greater, than that found in animals such as the cat, dog or man after feeding protein (3).

Significance of acceleration of oxidations in the fertilized echinoderm egg, in the light of these facts. It is a well-known fact that the rate of

oxidations in the egg of the sea urchin is increased several fold as a result of fertilization, and Warburg (4) has further shown that this increase in rate of oxidation need not be followed by cell division for when eggs were placed in dilute solutions of phenylurethane after fertilization, cell division was inhibited while the increase in the rate of oxidations was not prevented. However, in some of the fertilized

TABLE 3

Paramecium starved 48 hours in tap water. Duration only 9 hours in order to eliminate any possible effects of cell division in fed lots. The total number of Paramecia were too few to give any marked difference in CO_2 production between fed and starved animals in spite of the fact that 4 cc. of the Paramecium suspension were used instead of 2 cc. as in the previous experiments. Other conditions same as in tables 1 and 2.

BOTTLE	TITRATED AT ONCE	TITRATED AT END OF 9 HOURS			
	Controls. No yeast added			Yeast added	
	1	2	3	4	5
	Blanks	4 cc. Paramecia + 3 drops tap water. Starved	4 cc. "native tap" + 3 drops tap water. No Paramecium	4 cc. Paramecia + 3 drops yeast. Fed	4 cc. "native tap" + 3 drops yeast. No Paramecia. Control
1	21.05	20.25	20.25	20.08	20.31
2	21.05	20.25	20.30	20.12	20.26
3			20.32	20.15	20.33
Average.	21.05	20.25	20.29	20.11	20.30

	cc.
Average number cc. n/100.3 HCl equivalent of CO_2 absorbed from air in bottle and 4 cc. "native tap." Difference between columns 1 and 3	0.76
Average number cc. n/100.3 HCl equivalent of CO_2 produced by dead yeast. Difference between columns 3 and 5	0.01
Average number cc. n/100.3 HCl equivalent of CO_2 produced by starved Paramecium. Difference between columns 2 and 3	0.04
Average number cc. n/100.3 HCl equivalent of CO_2 produced by fed Paramecium. Difference between columns 4 and 5	0.19
Greatest difference in duplicate titrations	0.07

eggs treated with phenylurethane, protoplasmic movements and nuclear changes occur to a greater or less extent, and under certain conditions monasters are formed and even attempts at cell division (5). Hence for the sea urchin's egg the question is not quite clear as to whether increase in the rate of oxidation is entirely independent of the mitotic process in one form or other. In Paramecium it is certain

that cell division does not necessarily accompany the marked rise in rate of oxidations after feeding. If the same degree of independence

TABLE 4

Paramecia transferred slowly to pure tap water and left to starve for 15 hours. Then concentrated to 30 cc. Four cubic centimeters of this suspension were used for each test. Counts showed that in each 4 cc. there were from 28,000 to 30,000 Paramecia or from fifteen to twenty times the number present in each 4 cc. of the suspension used in experiment, table 3. Fifteen cubic centimeters $Ba(OH)_2$ *were added to each bottle, otherwise procedure was the same as in experiment, table 3. A test using yolk of hen's egg as food instead of yeast is given in columns 6 and 7. The yolk suspension was made by diluting fresh yolk with twenty-five volumes of distilled water.*

BOTTLE	TITRATED AT ONCE	TITRATED AT END OF 8 HOURS					
	Controls. No yeast added			Yeast added		Fresh yolk of hen's egg added	
	1	2	3	4	5	6	7
	Blanks	4 cc. Paramecia + 3 drops tap water. Starved	4 cc. "native tap" + 3 drops tap water. No Paramecium	4 cc. Paramecia + 3 drops yeast. Fed.	4 cc. "native tap" + 3 drops yeast. No Paramecia. Control.	4 cc. Paramecia + 4 drops yolk. Fed.	4 cc. "native tap" + 4 drops yolk. No Paramecia
1	15.85	14.71	15.00	13.85	15.15	14.00	15.00
2	15.72	14.63	15.00	13.80	14.95	14.05	15.05
3	15.71	14.70	15.08	13.91	15.02	14.20	15.02
Average..	15.76	14.68	15.03	13.85	15.04	14.08	15.02

	cc.
Average number of cc. n/100.3 HCl equivalent of CO_2 absorbed from air in bottle and 4 cc. "native tap." Difference between columns 1 and 3	0.73
Average number cc. n/100.3 HCl equivalent of CO_2 produced by dead yeast. Difference between columns 3 and 5	0.01
Average number of cc. n/100.3 HCl equivalent of CO_2 produced by starved Paramecia. Difference between columns 2 and 3	0.35
Average number of cc. n/100.3 HCl equivalent of CO_2 produced by Paramecia fed with yeast. Difference between columns 4 and 5	1.19
Average number of cc. n/100.3 HCl equivalent of CO_2 produced by Paramecia fed with yolk. Difference between columns 6 and 7	0.94
Greatest difference in duplicate titrations	0.20

of acceleration of oxidations and cell division occurs in the sea urchin egg as in Paramecium, then the means by which the sperm or other agent increases the oxidations in the egg may be by *making available*

for assimilation and growth the deutoplasmic food (yolk) reserve of the cell, just as ingestion of food into the food vacuole of Paramecium makes possible assimilation and growth and hence doubling or trebling of rate of oxidations. That the causes for an increase in rate of oxidations in the sea urchin's egg after fertilization and in Paramecium after feeding are the same seems furthermore entirely probable, for the following reasons. It has been definitely shown that increase in permeability of the surface protoplasm of the egg follows fertilization, and while we have no such clear evidence as to changes in permeability in the *internal* plasma membranes of the egg (phase boundaries between different components of protoplasm, such as alveolar membranes) at the time of cleavage, yet these phase boundaries of the internal parts of the cell undoubtedly undergo similar changes in permeability. One of the fundamental properties of protoplasm is its development of internal surface. If we are able to apply, as seems very probable, the facts and conceptions of permeability derived from study of the outer cell surface to internal cell membranes, such as walls of the alveoli which are so common a feature of cytoplasmic structure, then we have gone a long way toward discovering a mechanism which will account in a rational way for many well-known but isolated facts. One of these is the differential distribution of substances in the cell, such as pigments and different solutes in different vacuoles of the same cell. The internal plasma membranes and phase boundaries evidently serve as the means of separating and preventing an intermixing of these solutes. Internal plasma membranes because of their peculiar permeability properties make possible cellular *differentiation of the cell solution*. If admixture of these substances (e.g., enzymes and their substrates) are made possible by an increase in permeability of these internal plasma membranes, then chemical changes are initiated. In this way it is easy to conceive of the internal, differentially semi-permeable plasma membrane system as being the main controlling mechanism for at least certain of the chemical and physical processes in the cell. From this viewpoint it is readily seen that the yolk or food reserve of the egg (echinoderm) may not differ in its relation to the metabolism of the egg in any essential respects from the food reserve and ingested food of Paramecium. Fertilization in the egg involves increase in permeability of the limiting plasma membranes around the yolk particles in the cytoplasm, which leads to digestion, absorption and synthesis of living protoplasm and hence increase in rate of oxidations just as occurs in Paramecium. In Paramecium and

still better known for Bursaria (a ciliate similar to Paramecium) it has been definitely shown that the permeability of different food vacuole membranes differs and further that the same plasma membrane differs in its degree of permeability at different times during the process of digestion and absorption of the food contents. Furthermore this permeability is in this case to some extent selective (6). If the plasma membranes of the food vacuoles of Bursaria possess these properties is it not very probable that similar capacities for change should be present in the internal membranes of the sea urchin egg and other cells?

The results from the study of Paramecium in this and the previous paper, together with the above interpretation of the possible rôle of internal plasma membranes in the echinoderm egg at the time of fertilization, place the phenomenon of the marked increase in oxidation in Paramecium after feeding and the similar increase in rate of oxidation in the sea urchin egg after fertilization on the same basis and subject to the same interpretation, thus making clear the significance of the several-fold increase in rate of oxidations of the sea urchin's egg after fertilization.

SUMMARY

1. Feeding a starving Paramecium with yeast or yolk of hen's egg increases the rate of CO_2 production from two to three times, thus confirming the previous results on the measurements of the rates of oxygen consumption.

2. This acceleration of the oxidations occurs in the absence of cell division. The process of cell division as such in all probability is not associated with any marked change in the rate of oxidations.

3. The immediate conditions which underlie the difference in rates of oxidation in the unfertilized and fertilized sea urchin's egg are in all probability the same as those underlying the difference in rate of oxidations in a starving and well-nourished Paramecium.

4. The results from experiments on Paramecium show that the method can be used for determining very small quantities of carbon dioxide produced by unicellular or other lower organisms.

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TWO NEW FACTORS IN BLOOD COAGULATION—HEPARIN AND PRO-ANTITHROMBIN

W. H. HOWELL AND EMMETT HOLT

From the Physiological Laboratory of the Johns Hopkins University

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A survey of the results of recent work indicates that at least six different substances are concerned in one way or another in the process of blood coagulation; namely, fibrinogen, thrombin, prothrombin, calcium, antithrombin and the so-called zymoplastic or thromboplastic substances furnished by the body cells in general, including the blood corpuscles. With regard to the last mentioned factor satisfactory evidence has been obtained to show that the active material in tissue extracts is a phosphatid (1) (cephalin). Work that has been going on in this laboratory during the past two or three years shows that we must add two other substances to this list of fibrin factors. So far as we know, neither of these substances has been recognized by other workers.

The two new factors are: First, a phosphatid, not previously described, which exists in various tissues but is found in greatest abundance in the liver. This phosphatid is designated as *heparin*[1] to indicate its origin from liver. It inhibits coagulation, partially or completely according to the concentration. Second, a substance present in blood plasma and blood serum which is converted into antithrombin by a reaction with heparin. Just as in plasma and serum there is a mother substance from which thrombin is formed, so there is an antecedent substance from which antithrombin is formed. By analogy this substance is designated as pro-antithrombin. Prothrombin is activated to thrombin by calcium, the pro-antithrombin is activated to antithrombin by heparin.

Preparation of heparin. Attention was first called to this substance during some work done in this laboratory by Jay McLean (2). In the

[1] In a previous publication—Harvey Lectures, 1916–17, Series xii—this substance was described under the name of *Antiprothrombin.*

course of his work McLean prepared cuorin from heart muscle by the method of Erlandsen (3) and attempted to prepare heparphosphatid from the liver by the method of Baskoff (4). The cuorin obtained corresponded in all of its properties and in its N to P ratio with the material described by Erlandsen. When freed from contamination with cephalin the cuorin in solution in sodium chloride, 0.9 per cent, was found to have an inhibiting action on clotting. The phosphatid obtained from the liver did not correspond in its properties or in its N to P ratio with the heparphosphatid described by Baskoff. When freed from mixture with cephalin, a result most difficult to accomplish, this substance had a very marked inhibiting effect upon coagulation of blood, its action in this respect being much stronger than that of cuorin. We have varied the method of preparation in many different ways in the hope of devising a rapid and economical process for the isolation of this inhibiting phosphatid. The method finally selected, while yielding a reliable preparation of heparin, is time-consuming and expensive in material. The main obstacle lies in the fact that the solubilities of heparin and cephalin are so similar that there is great difficulty in separating them. But until the cephalin or cephalin-like material is removed the properties of the heparin, so far as coagulation is concerned, are masked or antagonized. The method of preparation finally adopted is as follows:

Fresh livers, preferably from dogs, are perfused, when possible, through the portal vein with a solution of sodium chloride, 0.9 per cent. They are then ground to a pulp, spread thin on a glass plate and dried in a current of hot air. The dried material is pulverized and extracted with ether in the proportion of 300 cc. of ether to each 100 grams of dried liver. The extraction may be made in a few hours with a shaking machine or by allowing the mixture to stand for 24 to 48 hours with an occasional shaking. The extract is then filtered and refiltered in a closed space until the filtrate is free from turbidity. The dark red filtrate is evaporated to dryness before an electric fan, and the sticky residue is treated with water-free ether (Squibb's) in as small quantities as suffice to dissolve it. There is always in this extract an insoluble residue which must be centrifugalized off. The clear red extract is then precipitated by acetone, the extract being added slowly with stirring to four times its volume of acetone. The precipitation is done in centrifugal tubes and the mixture is then centrifugalized. The supernatant liquid is drained off and discarded. The residue is dried in a current of air to drive off the adherent acetone. The residue is again dissolved in water-free ether, using as little ether as possible, and the solution is centrifugalized for a few minutes and poured off from any residue that may be obtained. The ether solution is then added slowly with stirring to four times its volume of absolute alcohol at 50°C. The mixture is kept in the warm bath for some time until the precipitate has settled. The supernatant liquid is decanted as far as

possible and the balance is poured into centrifugal tubes and centrifugalized while warm. The supernatant liquid is poured off and the residue is dried in a current of warm air until the excess of alcohol is driven off. The residue is again dissolved in water-free ether, centrifugalized, precipitated at 50°C. by four volumes of absolute alcohol and so on as described above. This procedure is repeated until the precipitate formed by the absolute alcohol begins to lose its solubility in water-free ether. This change in solubility of the alcohol precipitate takes place quite suddenly sometimes after twelve to twenty precipitations, but sometimes more gradually. When, after at least two hours treatment with ether, the residue on centrifugalization is suddenly increased in amount, the looked-for change is taking place. Thereafter each such residue is preserved. It is first washed with fresh ether and is then dissolved in water-free chloroform (Squibb's). The chloroform solution of the material is given two final precipitations. First, it is precipitated by two volumes of absolute alcohol at 50°C. This precipitate is separated by centrifugalization and is again treated with chloroform. After the material is completely dissolved the solution is precipitated, this time at room temperature, by the addition of an equal volume of absolute alcohol. The precipitate is centrifugalized off and dried in a current of warm air. The dark residue is ground in a mortar to a chocolate colored powder and kept until needed in a desiccator. This material should be completely soluble in water giving, according to concentration, a clear brownish or red solution, with no indication of opalescence. Material kept now for two years shows no deterioration in its property of preventing coagulation.

Properties of heparin. As stated above the material is soluble in water without opalescence. The occurrence of opalescence in the solutions is an indication of admixture with cephalin and if much of the latter is present the inhibiting effect on coagulation is lessened or absent altogether in freshly made preparations. When such preparations are kept for some weeks or months it will be found that the inhibiting action of their solutions is greatly improved. The explanation of this change is found in the fact that cephalin exposed to air loses gradually its favoring action upon coagulation (5).

The mode of preparation of heparin indicates that it belongs to the group of phosphatids—it gives reactions for nitrogen and phosphorus. Through the kindness of Doctor Levene of the Rockefeller Institute analyses for N and P were made in two samples. As these samples were made a year apart the mode of preparation was slightly different in the two cases but both preparations gave clear, non-opalescent solutions which exhibited a very marked inhibiting effect upon coagulation and when added to plasma or serum caused the formation of antithrombin. The results of these analyses were as follows:

Preparation I—March, 1917

Nitrogen	5.01 per cent
Phosphorus	4.75 per cent
Ratio of nitrogen to phosphorus	2.4 to 1

Preparation II—March, 1918

Nitrogen	5.92 per cent
Phosphorus	4.93 per cent
Ratio of nitrogen to phosphorus	2.6 to 1

The preparations had therefore a fairly constant composition in nitrogen and phosphorus, the ratio of one to the other being probably 2.5 to 1.

As compared with the phosphatids that have been obtained from the liver, heparin resembles most some of the preparations of jecorin that have been described. For his preparations of jecorin, Baskoff gives a mean value of the N to P ratio of 2.38 to 1, but in the preparations of other workers this ratio has varied from 1.8 to 1 (Drechsel) to 6.08 to 1 (Siegfried and Mark). For his preparations of heparphosphatid, Baskoff gives the N to P ratio as approximately 1 to 1.5. The two characteristic reactions of heparin that are of special physiological interest are first, that it retards or prevents the coagulation of blood; and second, when added to plasma or serum it causes the formation of a notable amount of antithrombin. Both reactions have been studied as carefully as possible.

The inhibiting action on coagulation. This effect may be demonstrated on the shed blood or upon the circulating blood by intravascular injections. The solutions of heparin used were made up in 0.9 per cent sodium chloride to prevent hemolysis of the blood corpuscles. In shed blood it was found that in mixtures containing 0.1 per cent of heparin the coagulation was prevented entirely. In more dilute solutions the coagulation was delayed in proportion to the concentration. When injected intravenously kymographic records showed that there was no disturbance of heart rate, blood pressure or respiratory rhythm and no variation in rectal temperature. If the injection of heparin is made in the proportion of one decigram per kilogram of body weight, blood drawn within a few minutes after the injection shows no spontaneous coagulation. Later the effects of the injected heparin wear off gradually so that in three or four hours the coagulation time of the blood has returned to normal. The gradual wearing off of the effect of the injected heparin was shown also in the variations of the prothrombin

time (6)—that is to say, the time of coagulation of the oxalated plasma when recalcified with the optimum amount of calcium chloride. Thus in one of the earlier experiments the following figures were obtained:

Prothrombin time before injection—dog	5 minutes
Prothrombin time 10 minutes after injection	No clot in 24 hours
Prothrombin time 1 hour after injection	38 minutes
Prothrombin time 2 hours after injection	14 minutes

Experiments made to determine how the heparin acts in preventing coagulation gave decisive proof that it inhibits the activation of prothrombin to thrombin. Its action certainly is entirely different from that of hirudin. This latter substance is a true antithrombin—that is to say, when added in minute amounts to mixtures of thrombin and fibrinogen it prevents clotting. Heparin has no such effect. Added in relatively large amounts to solutions of thrombin it has no retarding effect upon the action of the thrombin on fibrinogen. Since the heparin has no action upon fully formed thrombin and since it prevents the formation of thrombin when oxalated plasma is recalcified, it follows that the heparin must prevent the activation of prothrombin to thrombin, probably either by a reaction with the prothrombin or by a reaction with calcium. There are no facts to support the latter view. Heparin gives no precipitate with calcium salts and no indication of combining with the calcium in any way. Blood that has been rendered incoagulable with heparin cannot be made to clot by adding calcium chloride in any amounts. For example: in one experiment blood was caught as it flowed from an artery into a solution of heparin so that the concentration of the mixture was 0.15 per cent in heparin. This mixture was centrifugalized and the clear plasma was removed. This plasma remained unclotted as long as it was observed (24 hours). Mixtures were made with a solution of calcium chloride 0.5 per cent in the proportions of 5 drops of plasma to 1, 2, 3, 4 and 8 drops of the calcium chloride. None of these mixtures, or of similar mixtures in which the calcium chloride was replaced by water, showed any signs of clotting within 24 hours. The probability therefore is that the heparin reacts in some way with the prothrombin to prevent its activation by calcium. This conclusion is supported by direct experiments with solutions of prothrombin. Repeated experiments of this character were made with prothrombin prepared from the oxalated plasma of cat's blood (7). For example:

Prothrombin solution	5 drops
Heparin (0.2 per cent)	2 drops
Calcium chloride (0.5 per cent)	2 drops
Fibrinogen	8 drops

This mixture gave no clot in 24 hours—whereas a control mixture in which the heparin was replaced by an equal amount of water gave a solid clot within 5 minutes.

Heparin is a stable compound. As stated above, preparations kept in desiccators have retained their properties unimpaired for at least two years. Its solutions are not injured by heating to the boiling point. Specific experiments of this kind have been made with solutions containing heparin in concentrations of 0.5, 0.25 and 0.12 per cent. The test tubes containing these solutions were immersed for five minutes in boiling water. Subsequent examination showed that the activity of these solutions in preventing clotting and in activating pro-antithrombin had not been impaired.

The activation of pro-antithrombin. Early in this work it was observed that while heparin itself does not act as an antithrombin nevertheless it causes a marked increase in the antithrombin of plasma or serum. The following experiment may be used to illustrate this reaction. Clear oxalated and centrifugalized plasma from cat's blood was heated to 54°C. in a water bath to remove its fibrinogen. The precipitated fibrinogen was removed by filtration and the plasma was then tested for antithrombin by the method used in this laboratory (8) with and without the addition of a solution of heparin. One drop of each solution was added to 2, 3, 4 and 5 drops of thrombin and after an incubation of 15 minutes 10 drops of fibrinogen were added to each specimen and the time of coagulation was noted.

Specimen I. Plasma plus an equal volume of 0.1 per cent heparin

Thrombin 2 drops	No clot in 24 hours
Thrombin 3 drops	Clot in 95 minutes
Thrombin 4 drops	Clot in 42 minutes
Thrombin 5 drops	Clot in 17 minutes

Specimen II. Control—Plasma plus an equal volume of water

Thrombin 2 drops	Clot in 17 minutes
Thrombin 3 drops	Clot in 5 minutes
Thrombin 4 drops	Clot in 4 minutes
Thrombin 5 drops	Clot in 3 minutes

As will be seen, the specimen containing heparin showed a greatly increased amount of antithrombin. This experiment was repeated a great many times with specimens of serum as well as of plasma and always with similar results. In attempting to explain this reaction it was at first supposed that the heparin reacted with some of the known substances in serum to produce an antithrombin, for example, with prothrombin or antithrombin itself, or that in some way it sensitized or intensified the action of the antithrombin already present in the plasma or serum (9). Experiments made to test these hypotheses have given negative results. Prothrombin can be isolated in an impure but effective form by the acetone method. Solutions of this kind have no antithrombic action and addition of heparin does not alter the reaction in this respect. On the other hand it was found that the antithrombin in cat's plasma can be removed or destroyed by heating to 60°C. for five to ten minutes. Nevertheless on adding heparin to such a plasma there is an immediate production of a considerable amount of antithrombin. Hence the heparin does not act by intensifying the activity of antithrombin already present but produces new antithrombin by a reaction with an unknown constituent of the blood. This conclusion is strengthened further by the fact that this antecedent or mother substance can be precipitated from plasma by one-third or one-half saturation with ammonium sulphate. The precipitation by one-third saturation is less complete that with one-half saturation but in neither case is the active antithrombin thrown down, or if it is precipitated it is destroyed or denatured in the process. For example: a specimen of cat's plasma, oxalated and centrifugalized, was divided into two portions; to one portion ammonium sulphate was added to one-third saturation and to another to one-half saturation. The precipitates were collected by centrifugalizing and were then dissolved by the addition of water. Each solution was tested for antithrombin after adding an equal volume of heparin (0.2 per cent) and, as a control, after adding an equal volume of water, with the following result:

I. The precipitate obtained by one-third saturation with ammonium sulphate

With heparin

Thrombin 2 drops	No clot in 1 hour
Thrombin 3 drops	No clot in 1 hour
Thrombin 4 drops	Clot in 53 minutes
Thrombin 5 drops	Clot in 15 minutes
Thrombin 2 drops	No clot in 1 hour

With water (control)

Thrombin 2 drops	Clot in 4.5 minutes
Thrombin 3 drops	Clot in 3.5 minutes
Thrombin 4 drops	Clot in 2.5 minutes
Thrombin 5 drops	Clot in 2.5 minutes

II. The precipitate obtained by one-half saturation with ammonium sulphate

With heparin

Thrombin 2 drops	No clot within an hour
Thrombin 3 drops	No clot within an hour
Thrombin 4 drops	No clot within an hour
Thrombin 5 drops	No clot within an hour

With water (control)

Thrombin 2 drops	Clot in 4 minutes
Thrombin 3 drops	Clot in 3 minutes
Thrombin 4 drops	Clot in 2.5 minutes
Thrombin 5 drops	Clot in 2 minutes

These results can scarcely be interpreted otherwise than upon the assumption that there is a material in plasma and serum which by a reaction with heparin is converted to or gives rise to antithrombin. In the nomenclature of the day we may speak of this substance as a pro-antithrombin which is activated to antithrombin by heparin, just as prothrombin is activated to thrombin by calcium.

The two sets of compounds show an interesting difference in their reactions to acetone. The precipitate obtained by adding an equal volume of acetone to blood plasma yields on appropriate treatment an active prothrombin but no pro-antithrombin. So also from solutions in which it is contained thrombin may be precipitated uninjured by acetone while antithrombin is destroyed or denatured. On the other hand ammonium sulphate to one-half saturation precipitates uninjured prothrombin, thrombin and pro-antithrombin, while antithrombin is either not precipitated or else is destroyed or denatured. It was observed also that in cat's oxalated plasma, after heating to 54°C. to remove the fibrinogen, both prothrombin and pro-antithrombin may be precipitated in part by the addition of acetic acid to distinct acid reaction. The precipitate may be collected by centrifugalizing and then dissolved in a solution of sodium chloride, 1 per cent, made slightly alkaline with sodium carbonate. This solution furnishes thrombin on the addition of calcium chloride and antithrombin on the addition of heparin.

The pro-antithrombin or antithrombogen, as it might also be called, is thermolabile but less so than antithrombin. As stated above, this

difference is especially well shown in cat's plasma. In this plasma all the ready formed antithrombin may be destroyed by heating to 60° for 5 minutes while the pro-antithrombin is apparently not affected. Heating this plasma to 70°C. however will destroy all the pro-antithrombin very promptly, and the same result is obtained by prolonged heating at lower temperatures. The following results were obtained from the plasma of cat's blood, oxalated and centrifugalized, subsequently heated to 60° for 5 minutes and 15 minutes and to 70° for 5 minutes and then examined for antithrombin with and without the addition of heparin.

I. Specimen heated to 60°C. for 5 minutes

With heparin	
Thrombin 2 drops	No clot in 24 hours
Thrombin 3 drops	No clot in 24 hours
Thrombin 4 drops	No clot in 24 hours
Thrombin 5 drops	No clot in 24 hours
With water (control)	
Thrombin 2 drops	Clot in 4.5 minutes
Thrombin 3 drops	Clot in 3.5 minutes
Thrombin 4 drops	Clot in 3.5 minutes
Thrombin 5 drops	Clot in 3.5 minutes

II. Specimen heated to 60°C. for 15 minutes

With heparin	
Thrombin 2 drops	Clot in 32 minutes
Thrombin 3 drops	Clot in 10 minutes
Thrombin 4 drops	Clot in 10 minutes
Thrombin 5 drops	Clot in 5 minutes

III. Specimen heated to 70°C. for 5 minutes

With heparin	
Thrombin 2 drops	Clot in 4.5 minutes
Thrombin 3 drops	Clot in 3.5 minutes
Thrombin 4 drops	Clot in 2.5 minutes
Thrombin 5 drops	Clot in 2.5 minutes

It will be seen that the reaction with heparin was much weakened by heating to 60°C. for 15 minutes, and completely destroyed by heating to 70° for 5 minutes.

The source of pro-antithrombin. An effort was made to determine whether or not the pro-antithrombin is furnished to the plasma by the blood corpuscles. The experiments were not completed but they were carried far enough to show that neither the blood plates nor the leucocytes give origin to this substance. Platelets obtained from oxalated

pig's blood by differential centrifugalization and subsequent washing with a 0.9 per cent solution of sodium chloride give a solution when extracted with water which contains prothrombin but no pro-antithrombin—that is to say, addition of calcium chloride causes the formation of thrombin, but addition of heparin does not produce antithrombin. A similar negative result, so far as pro-antithrombin is concerned, was obtained by extracting lymphocytes furnished by the thymus gland and blood leucocytes obtained from the buffy coat of centrifugalized oxalated blood.

The reaction between heparin and pro-antithrombin. We have no knowledge regarding the chemical nature of pro-antithrombin or the change that it undergoes in being converted to active antithrombin, but it seems evident that the reaction is not enzymatic or catalytic. The amount of antithrombin formed increases in proportion to the amount of heparin added. If one takes several specimens of oxalated plasma of 0.5 cc. each and adds one, five and ten drops of a 0.2 per cent solution of heparin it will be found that the amount of antithrombin formed increases with the heparin added. Unfortunately the mode of demonstrating antithrombin does not lend itself readily to a quantitative determination, except within narrow limits, so that it is not possible to express the relationship between the amounts of heparin and the amounts of antithrombin in a definite form. But the fact that the reaction between the heparin and the pro-antithrombin is of the nature of a definite chemical reaction is borne out also by the following observation. A specimen of clear cat's plasma, oxalated, was heated to 60°C. for ten minutes, thereby precipitating its fibrinogen and destroying all the antithrombin present. After filtration, a 0.2 per cent solution of heparin was added in equal volume to a part of this plasma. Examination showed now the presence of a considerable amount of antithrombin, due to the action of the heparin on the pro-antithrombin. This mixture was then heated again to 60°C. for ten minutes to destroy the newly formed antithrombin, and that this really occurred was determined by experiment. To the mixture there was again added an equal volume of heparin, 0.2 per cent, with the result that there was again a formation of a considerable amount of antithrombin. The experiment serves not only to indicate that the reaction between the heparin and pro-antithrombin takes place in definite proportions after the manner of an ordinary chemical reaction, but it serves to show also that the reserve supply of pro-antithrombin in blood is considerable. The reaction between antithrombin and thrombin, as has been pointed

out previously (10), is a colloidal reaction the extent of which varies with the time, so that in any effort to estimate the amount of antithrombin in a quantitative way in different solutions it is necessary to give a definite time for the interaction between the two substances. The longer they are in contact the larger will be the amount of thrombin inactivated by the antithrombin. The relations are quite different in the reaction between heparin and pro-antithrombin. When a mixture is made the maximal reaction is completed as soon as an examination can be made, and a longer exposure leads to no further increase in antithrombin.

DISCUSSION

One, at least, of these new factors in coagulation, the pro-antithrombin, is present normally in blood. The other, the heparin, has not been shown directly to be a normal constituent of blood but the indirect evidence speaks strongly in favor of this view. The direct rôle of the pro-antithrombin is to give rise to antithrombin and it may be assumed that the amount of the latter constantly present in normal blood is dependent upon the extent to which the pro-antithrombin is activated, and this in turn is dependent upon the amount of heparin since so far as we know the pro-antithrombin cannot be changed to antithrombin otherwise than by a reaction with heparin. The fact that there is a supply, and a considerable supply, of pro-antithrombin in circulating blood indicates that the antithrombin probably takes an important part in regulating the process of coagulation. What is the rôle of antithrombin? Two views have been suggested: Nolf (11) and afterwards Howell (12) have stated their belief that the antithrombin is directly concerned in maintaining the normal fluidity of the blood. The latter in his theory of coagulation has assumed that some inhibiting substance, presumably the antithrombin, functions by preventing the activation of prothrombin by calcium and that in this way intravascular coagulation is avoided. When blood is shed or when in any other way thromboplastic substance is added to the blood, the inhibiting influence of the antithrombin is neutralized, the prothrombin is converted to thrombin and coagulation ensues. At the time this theory was proposed antithrombin was the only inhibiting substance known in the blood, but the work described in this paper makes it probable that the blood contains a second inhibiting substance, heparin, which so far as its action is concerned, may be designated as an anti-prothrombin since it inhibits clotting by preventing

the activation of prothrombin. It would be more logical perhaps to assume that heparin rather than antithrombin is the substance in blood which prevents spontaneous clotting and which is neutralized in shed blood by the thromboplastic material, the cephalin. Laboratory experiments show readily that the inhibiting effect of heparin can be neutralized by the addition of cephalin, but in order to give this modification of Howell's original theory a sound experimental basis it is necessary to show that heparin is a normal constituent of blood. This demonstration has not yet been furnished. But admitting the probability of this view the question arises as to the function fulfilled by the antithrombin which we know does exist in normal blood. In several pieces of work done in this laboratory (Weymouth, 13), (Gasser, 14), (Rich, 15), evidence has been furnished which tends to show that metathrombin found in the serum of clotted blood is a combination of thrombin and antithrombin. The antithrombin inactivates thrombin by forming the substance designated as metathrombin by Morawitz. In accordance with this view Gasser has suggested that this may be at least one function of the antithrombin. It is very probable that thrombin in small amounts is formed constantly in circulating blood and the presence of antithrombin may be a device to neutralize this thrombin. On the other hand, when blood is shed thrombin is formed suddenly in relatively large amounts and the antithrombin under such conditions is inadequate for protection, especially as its rate of action upon thrombin is greatly reduced at temperatures below that of the body (16). Provisionally we are inclined to adopt the view that the value of the antithrombin and therefore of the pro-antithrombin is to safeguard the blood from the action of any free thrombin that may be liberated in the circulation.

The function of the heparin in coagulation is implied in its two characteristic properties described above. As previously stated, the presence of this substance in the blood has not been demonstrated but it seems very probable that it is one of the normal constituents of this liquid. The fact that it has the specific reaction of changing pro-antithrombin to antithrombin supports this view. If we can make this assumption it would follow that heparin constitutes an inhibiting material which restrains clotting by a double action; by its power to prevent the activation of prothrombin to thrombin and by its property of activating pro-antithrombin to antithrombin. Through this material the body is able to modify coagulability of the blood either locally or in general. An instance of local modification is furnished by the

work of Schickele (17) who has shown that an inhibiting substance to blood coagulation is produced in the uterine mucous membrane. A special study of this material in this laboratory by Dr. Jessie King has shown that it is undoubtedly heparin or a substance with properties similar to those of heparin, since it inhibits clotting by preventing the activation of prothrombin and is also effective in causing an increase in antithrombin when added to plasma or serum. It is quite possible indeed that some of the normal and abnormal variations in the coagulability of blood may be traced to variations in the amount of heparin. The properties of hemophilic blood, for example, as described by one of us (18); are in fact identical with those exhibited by blood to which heparin has been added in concentrations sufficient to retard coagulation. If subsequent work is successful in devising a method for the quantitative estimation of heparin in blood, a great amount of light may be thrown upon some of the difficult problems in blood coagulation.

SUMMARY

1. Two new substances are described which are connected with the process of coagulation of blood:

a. A phosphatid designated as heparin since it is obtained most readily from the liver (dog). It has an N to P ratio of 2.5 to 1. It is easily soluble in water giving a clear, non-opalescent solution of a yellowish or reddish color according to concentration. It possesses two characteristic reactions: First, it retards or prevents the coagulation of blood, both in the body and when the blood is shed. Second, it causes a marked increase in antithrombin when added to blood or serum.

b. An antecedent or mother substance for antithrombin, present in plasma and serum in considerable amounts. It is converted promptly to antithrombin by heparin and is designated as a pro-antithrombin.

2. Heparin may be obtained from lymph glands and it has been detected by its reactions in the uterine mucous membrane. It is present in largest amounts in the liver, especially the dog's liver.

3. Heparin inhibits clotting mainly by preventing the activation of prothrombin to thrombin. It acts as an antiprothrombin rather than as an antithrombin.

4. The action of heparin in preventing the activation of prothrombin and in causing the activation of pro-antithrombin is not destroyed at a temperature of 100°C.

5. Pro-antithrombin in blood plasma is destroyed promptly by heating to 70°C. and more gradually by prolonged heating at lower temperatures (60°C.).

6. Pro-antithrombin may be precipitated uninjured from blood plasma by acetic acid or by ammonium sulphate added to one-half saturation.

7. On the theoretical side it is suggested that heparin and pro-antithrombin are normal constituents of the circulating blood (not demonstrated for the heparin) and together fulfill the function of safeguarding the fluidity of the blood, that is to say, of preventing intravascular clotting. The pro-antithrombin by its conversion to antithrombin provides a protection against any small quantities of thrombin that may arise in the circulating blood (metathrombin formation). The heparin in addition to functioning as a specific activator to pro-antithrombin exerts an inhibiting influence upon the conversion of prothrombin to active thrombin. When blood is shed or when in other ways thromboplastic substance (cephalin) is added to the blood the protection afforded by the heparin is overcome and thrombin is formed in amounts sufficient to cause clotting. It is suggested that variations in the amount of heparin in the blood may suffice to explain some of the known abnormalities in coagulation, hemophilia, for example.

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THE INFLUENCE OF SECRETIN ON THE NUMBER OF RED AND WHITE CORPUSCLES AND THE FERMENTS AND SUGAR CONTENT OF THE BLOOD

B. FUJIMOTO

From the Forensic-Medical Institute of the Imperial University at Tokyo

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In 1902 Bayliss and Starling (1) showed that acid acts on prosecretin in the duodenal mucous membrane and changes it into secretin, which is carried by the blood and activates the pancreatic cells. They showed also that secretin increases the secretion of bile.

But as to its effects on the internal secretion no researches have apparently been carried out, except the fairly recent work of Downs and Eddy, (2), (3) who performed several interesting experiments and found that the subcutaneous injection of even a small dose of secretin is able to produce a marked increase in the number of red and white corpuscles in the circulating blood. They suggested as the most probable explanation of this increase that it is due to a direct stimulating action of the secretin on the bone marrow and lymphatic tissues in general.

In our laboratory more extensive researches in this field have been carried out, namely: the influence of secretin on several ferments (catalase, diastase and glycolytic ferment) and on the sugar content of the blood.

The secretin which we used was always prepared as follows: Dogs weighing about 10 kgm. each were used. They received no food for about twenty-four hours previous to being bled. The mucous membrane was scraped off with a dull knife from the duodenum and the upper half of the small intestine, and was triturated with 50 cc. of 0.4 per cent hydrochloric acid. After standing for two hours, the mixture was boiled actively, neutralized with 10 per cent solution of sodium hydroxide while boiling and again rendered faintly acid with glacial acetic acid. After boiling for about ten minutes the preparation was filtered. The filtrate thus obtained was clear and greenish-yellow. Sufficient glacial acetic acid was added to make it 2 per cent by volume

and the acid extract evaporated to dryness, as was done by Downs and Eddy. We thus obtained about 0.5 to 0.7 gram of dried acid extract, namely about 10 to 14 mgm. per cubic centimeter of original solution.

In our experiments the dried acid extract was dissolved in normal saline solution and injected into rabbits subcutaneously. In all experiments recorded in this paper rabbits were employed. During the experiments food and water were always withheld from the animals.

The investigations to be described were commenced with the repetition of Downs and Eddy's work.

The influence of secretin on the number of red and white corpuscles in the circulating blood. Downs and Eddy have shown that the administration of secretin produces a considerable increase in both the red and the white corpuscle count. Our experiments were carried out in accordance with their procedure. The blood was obtained from the ear of the rabbit. Specimens were taken simultaneously for counting both red and white corpuscles, Hayem's solution being used as the diluting fluid for the former and Türk's solution for the latter. The counts were made in the usual manner with the Thoma-Zeiss apparatus.

One cubic centimeter of the secretin solution, equivalent to approximately 10 mgm. of the dried acid extract, per kilogram of body weight, which was determined by Downs and Eddy as the most efficient dose to produce a marked increase in the number of red and white corpuscles per cubic millimeter of the circulating blood, was injected subcutaneously. The results are shown in table 1.

These experiments show that the secretin solution, when injected subcutaneously, is able to produce an increase in the number of red and white corpuscles in the circulating blood thus confirming the results of Downs and Eddy.

The influence of secretin on the catalase content of the blood. The object of this investigation was to determine what change, if any, occurs in the catalase content of the blood. The amount of catalase in the blood was determined according to the following method.

The blood was obtained in all cases from the ear of the rabbit with as little manipulation as possible, and quickly defibrinated. One cubic centimeter of this defibrinated blood was diluted with normal saline solution to 0.1 per cent. Ten cubic centimeters of this solution of the blood were added to 30 cc. of 1 per cent neutral hydrogen peroxide in a specially arranged flask. The liberated oxygen gas was conducted through a rubber tube to a graduated burette, which was previously filled with water. As the liberation of the oxygen is consider-

ably accelerated by shaking the flask and therefore the amount of the liberated gas may vary with such shaking, we have, as a rule, never touched the apparatus after the blood specimen and hydrogen peroxide were mixed. Exactly after twenty minutes, the atmospheric pressure in the burette was made equal to that in the room and the amount of

TABLE 1

Dose: 1 cc. secretin solution per kilogram of body weight

EXPERIMENT NUMBER		INITIAL COUNT	MAXIMUM COUNT	AMOUNT OF INCREASE	PERCENTAGE INCREASE	MAXIMUM IN
						minutes
1	R. B. C.	5,550,000	7,030,000	1,480,000	27	60
	W. B. C.	10,100	10,700	600	6	60
2	R. B. C.	5,110,000	6,780,000	1,670,000	33	90
	W. B. C.	15,200	23,300	8,100	19	90
3	R. B. C.	5,870,000	7,030,000	1,160,000	20	60
	W. B. C.	6,100	9,000	2,900	38	60
4	R. B. C.	5,720,000	7,640,000	1,920,000	34	90
	W. B. C.	11,000	16,200	5,200	47	120
5	R. B. C.	6,130,000	10,070,000	3,940,000	64	90
	W. B. C.	5,200	10,200	5,000	96	90
6	R. B. C.	6,060,000	6,970,000	910,000	15	90
	W. B. C.	6,400	8,500	2,100	33	90
7	R. B. C.	5,940,000	6,420,000	460,000	8	90
	W. B. C.	9,000	11,300	2,300	26	90
8	R. B. C.	6,240,000	6,560,000	320,000	5	90
	W. B. C.	7,900	8,100	200	3	90
Average percentage increase	R. B. C.					25.75
	W. B. C.					34.75

the oxygen was read off directly from the burette in which the gas had displaced the water. The volume of oxygen gas which is liberated by 1 cc. of the blood in a minute was taken as a measure of the amount of catalase in the defibrinated blood.

Burge (4), (5) has reported the effect of the emotions on the catalase content of the liver and blood. He has also shown that the catalase

content of the blood is increased by prolonging the excitement stage of ether anaesthesia and believes that the increase in catalase may account for the increased oxidation during the excitement stage of ether anaesthesia just as the decrease in catalase may account for the decreased oxidation during the subsequent stages of anaesthesia. Burge and Neill (6) also demonstrated that the catalase of the blood is decreased in the condition of "shock." In our preliminary experiments dealing with the effect of secretin on the blood catalase in rabbits it was found that the injection caused an actual decrease in the catalase content. A similar decrease was, however, noted in control animals. Subsequently it was discovered, as is indicated in table 2, that this

TABLE 2

The catalase content of normal rabbits

RABBIT NUMBER	DATE	CATALASE					
		In the beginning	After				
			30 minutes	60 minutes	90 minutes	120 minutes	150 minutes
6	May 22	67.0	45.0	41.0	37.0		
7	May 23	55.0		52.5	45.0	44.0	44.0
8	May 24	100.0	95.5	89.5	87.0	84.0	82.5
9	May 24	33.0	31.5	30.0	28.5	30.5	
6*	May 25	29.0		48.0	38.5	34.5	
1*	May 25	73.0		80.0	80.0	73.0	
10	May 27	90.0		87.0	89.0	86.5	86.0
11*	May 27	82.0		86.0		74.5	65.0
7	May 28	71.5		65.5	65.5	75.0	72.5
8*	May 28	71.5		74.0	73.0	77.0	

decrease was apparently associated with the handling and fright of the animals for if time was allowed to elapse, the catalase determinations became reasonably constant.

Four experiments marked with an asterisk (*) show an increase in the catalase content at first. Probably these animals were less frightened by repeating the experiment. As we see in the foregoing table, the catalase content in the blood decreases mostly during the beginning one and a half hour of the experiments and then becomes relatively constant.

In our experiments secretin was, therefore, injected hypodermically after the catalase content became relatively constant. One cubic

centimeter of secretin per kilogram of body weight was given hypodermically. The results obtained will be found in table 3.

Experiments 4 and 9 showed no increase in the catalase content. In experiment 1 the secretin was injected intravenously. Except for these experiments, a dose of 1 cc. of secretin solution per kilogram of body weight was able to produce an average increase of 17 per cent in the catalase content of the circulating blood. It was not determined whether there is a more effective dose than the one used.

Several experiments for control were undertaken in order to determine if this increase in the catalase content of the blood is caused

TABLE 3

Dose: 1 cc. secretin solution per kilogram of body weight

EXPERIMENT NUMBER	RABBIT NUMBER	DATE	CATALASE						
			Before	Time after the injection of secretin					
				30 minutes	60 minutes	90 minutes	120 minutes	Percentage increase	Maximum in
									minutes
1	7	May 23	43.5	47.0	43.5	39.5	44.5	8	30
2	8	May 24	67.0	80.5	80.0	70.5	68.5	20	30
3	9	May 24	30.0	34.5	31.0	29.5	27.5	15	30
4	6	May 25	45.0	41.0	42.0	36.5		0	
5	1	May 25	53.0	71.0	80.0	67.0		50	60
6	10	May 27	81.0	83.0	88.0	90.0	76.5	11	90
7	11	May 27	69.0	72.0	80.0	73.0	69.5	11	60
8	8	May 28	77.0	81.0	75.0	76.0	70.0	5	30
9	7	May 28	75.0	72.5	66.5	64.0	68.5	0	

Average percentage increase, 17; maximum in 47 minutes

especially by the injection of secretin solution. At first, 2 or 3 cc. of normal saline solution were injected subcutaneously in rabbits, after the catalase content in the blood became relatively constant. But no increase in the catalase was seen, as the following table shows.

As the secretin solution contains peptone, we have proceeded to the further experiments for control in order to determine if the peptone itself has an effect on the catalase content of the blood. For this purpose we performed the following four experiments with a dose of 0.01 gram per kilogram of body weight. The peptone was dissolved in normal saline solution and injected hypodermically. The results are shown in table 5.

A slight increase was found in two experiments. But comparing these results with those of the experiments with secretin, the increase is insignificant. Hence the conclusion is justified that the hypodermic injection of the secretin solution causes a considerable increase in the catalase content of the blood in thirty to ninety minutes. Since catalase is found in the blood corpuscles, it is natural to infer a relationship

TABLE 4

Influence of the hypodermic injection of normal saline solution (2 or 3 cc.) on the catalase content in the blood

EXPERIMENT NUMBER	CATALASE			
	Before the injection	Time after the injection		
		30 minutes	60 minutes	90 minutes
1	34.5	32.5	32.0	31.0
2	44.0	44.0	44.5	43.5
3	77.5	80.5	71.0	67.0
4	33.0	32.0	30.5	30.0

TABLE 5

Experiments on the effect of peptone on the catalase content of the blood. Dose: peptone 0.01 gram per kilogram of body weight

EXPERIMENT NUMBER	CATALASE				
	Before the injection	Time after the injection			
		30 minutes	60 minutes	90 minutes	120 minutes
1	42.0	43.5	46.0	45.0	41.0
2	73.0	70.0	72.0	72.5	67.5
3	86.0	80.0	82.5	81.5	77.5
4	73.0	77.0	77.0	80.0	79.0

between the increased corpuscle count and the increase of catalase produced by the injection of secretin.

The influence of secretin on the diastase of the blood. Investigations were carried out to study the influence of secretin on several ferments in the blood. Diastase was first examined.

Inoue has recently made an interesting report on methods for the determination of diastase, which is not yet published. It was proved by him that Wohlgemuth's series method for the determination of diastase does not give reliable results. He has recommended a new

method, which gives uniformly good results. In our experiments this new method was employed; namely: normal saline solution, which contains a certain quantity of salt and acetic acid, was always used for the dilution of the blood serum. One cubic centimeter of the diluted serum solution (in our cases 5 per cent) was added to 1 cc. of 0.1 per cent amylum solution and incubated in a water bath at 38°C. At intervals of a minute a drop of Lugol's solution (1/50 N) was added to it to see whether the amylum was completely digested or not. The time which is necessary for the complete digestion of the amylum was taken as a measure of the amount of diastase. The results here recorded are based upon the examination of twelve different specimens obtained from eight different rabbits.

TABLE 6

EXPERIMENT NUMBER	THE TIME WHICH IS NEEDED TO DIGEST 1 CC. OF 0.1 PER CENT AMYLUM SOLUTION COMPLETELY BY 1 CC. OF 5 PER CENT SERUM SOLUTION				
	Before the injection	Time after the injection of secretin, 1 cc. per kilogram of body weight			
		30 minutes	60 minutes	90 minutes	120 minutes
	minutes	*minutes*	*minutes*	*minutes*	*minutes*
1	24	24	24	25	26
2	26	27	28	30	31
3	25	28	31	33	30
Control: nothing injected					
1	26	26	29	28	27
2	27	28	28	30	31

These experiments show that the content of diastase in the blood is not influenced at all by the hypodermic injection of the secretin solution in a dose of 1 cc. per kilogram of body weight. Incidentally we tested the serum of these rabbits, in which secretin had been injected, on the enucleated eyes of frogs to see if there was any adrenalin-like substance in the blood. The results were entirely negative.

The influence of secretin upon the sugar and the glycolytic ferment of the blood. The blood was taken from the ear of rabbits and quickly defibrinated. Four cubic centimeters were added immediately to 36 cc. normal saline solution, containing a known quantity of 6 per cent solution of pure dextrose. Twenty cubic centimeters of the mixture were examined immediately, and the remainder was placed in an incubator at 37°C. for twenty hours. The blood sugar content was

determined with the remaining portion of the defibrinated blood. Throughout the research rigid precautions as regards asepsis were taken.

The specimens were always precipitated by liq. ferri dialysat. and potassium sulphate (saturated solution), and filtered. In all estimations of sugar Pavy-Kumagawa-Sudo's method was employed. At first, 1 cc. of secretin solution per kilogram of body weight was given subcutaneously to see if it influenced the sugar and glycolytic ferment content of the blood. But, no influence being observed, 10 cc. per kilogram of body weight were administered. The results are shown in the following tables.

TABLE 7

EXPERIMENT NUMBER	SECRETIN	BLOOD SUGAR	
		Before the injection of secretin	One hour after the injection of secretin
	cc.	*per cent*	*per cent*
1	1	0.117	0.132
2	1	0.124	0.135
3	10	0.165	0.198
4	10	0.108	0.125
5	10	0.105	0.136

TABLE 8

EXPERIMENT NUMBER	SECRETIN		DEXTROSE			
			Immediately after	20 hours after at 36°C.	Loss of dextrose	Percentage loss
	cc.		*mgm.*	*mgm.*	*mgm.*	
1	1	Before injection	7.908	6.136	1.772	22.4
		One hour after	7.880	6.084	1.796	22.8
2	1	Before injection	8.020	5.156	2.864	35.7
		One hour after	8.020	5.342	2.678	33.4
3	10	Before injection	9.448	6.288	3.160	33.5
		One hour after	9.188	6.700	2.488	27.1
4	10	Before injection	8.772	5.460	3.312	37.8
		One hour after	8.732	5.648	3.084	35.3
5	10	Before injection	8.548	4.216	4.332	50.7
		One hour after	8.396	4.076	4.320	50.2

All of these experiments show some increase in the amount of the blood sugar. But, as the blood sugar content is labile, such a slight increase may be caused by factors such as excitement of the animal. It thus appears that the blood sugar is not influenced by the administration of secretin.

As we see in the foregoing table, no marked decrease in the glycolytic power of the blood was proved. It would thus appear that the sugar and glycolytic ferment of the blood are in no way altered quantitatively by the administration of secretin.

CONCLUSIONS

1. Secretin, when injected subcutaneously, produces an increase in the number of red and white corpuscles in the circulating blood of rabbits, as Downs and Eddy proved.

2. The hypodermic injection of 1 cc. of secretin causes an increase in the catalase content of the blood.

3. Neither increase nor decrease in the diastase, glycolytic ferment and sugar of the blood was observed after the hypodermic injection of 1 cc. of secretin.

I desire to express my sincere thanks to Prof. Dr. K. Katayama and Prof. Dr. S. Mita, to whom I am indebted for suggesting the problem and for their kind advice.

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THE INFLUENCE OF THE INGESTION OF THE AMINO-ACIDS, ADEQUATE AND INADEQUATE PROTEINS UPON THE PRODUCTION OF CATALASE

W. E. BURGE

From the Physiological Laboratory of the University of Illinois

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Lusk (1) found that the ingestion of glycocoll, alanin, leucin and tyrosin increased oxidation while the ingestion of glutamic acid did not. Grafe (2), contrary to Lusk, found that the ingestion of glutamic acid as well as glycocoll, alanin, leucin and tyrosin increased oxidation. We (3) had found that whatever increased oxidation in the body produced a corresponding increase in catalase by stimulating the digestive glands, particularly the liver, to an increased output of this enzyme, and that whatever decreased oxidation produced a corresponding decrease in catalase by decreasing the output from the liver and by the direct destruction of the enzyme in the blood and tissues. The object of the present investigation was to determine if the ingestion of amino-acids such as glycocoll and alanin would produce an increase in catalase parallel with the increase produced in oxidation, and whether amino-acids such as glutamic and aspartic would produce no increase in catalase in keeping with Lusk's contention that they do not increase oxidation, or whether they would produce an increase in catalase in keeping with Grafe's contention that the ingestion of these two amino-acids does increase oxidation. The effect of the ingestion of an adequate and of an inadequate protein was also studied. The adequate protein used was casein and the inadequate one, gelatin. A digest of each of these proteins was made by adding 200 cc. of activated pancreatic juice to 300 grams of each of the proteins in a small amount of a 1 per cent sodium carbonate solution. This material was kept in a thermostat at 40°C. for 200 hours. Thymol was the preservative used. The amounts of the amino-acids used were 5 grams per kilogram of body weight of the animal. The glutamic and aspartic acids were given in the form of the sodium salts and in concentrated solutions. The glycocoll and alanin were given in as concentrated

solutions as could be made with distilled water. The whole of the 300 grams of the protein digests was used.

After etherizing the animals and opening the abdominal wall, the materials were introduced into the intestine under pressure from a bottle through a rubber tube and an attached hypodermic needle. The catalase in 0.5 cc. of blood taken directly from the liver, the portal and jugular veins was determined before as well as at fifteen-minute intervals after the introduction of the different materials. The blood of the liver was collected from a superficial incision made in this organ. The catalase was determined by adding 0.5 cc. of blood to 50 cc. of diluted hydrogen peroxide in a bottle at approximately 22°C., and the amount of gas liberated in ten minutes was taken as a measure of the amount of catalase in the 0.5 cc. of blood.

In figure 1 under glycocoll, alanin, glutamic acid, aspartic acid, asparagin, (glutamic acid, glycocoll), (aspartic acid, glycocoll), (asparagin, glycocoll), are given curves, constructed from data obtained from dogs before as well as at fifteen-minute intervals after the introduction of these materials into the alimentary tract. The figures along the ordinate represent amounts of catalase measured in cubic centimeters of oxygen and the figures along the abscissae, time in minutes. The continuous line curves were constructed from data obtained from the blood of the liver, the discontinuous line curves from the blood of the portal vein, and the dotted line curves from the blood of the jugular. It may be seen in figure 1, dog 1, under glycocoll, that previous to the introduction of the glycocoll, 0.5 cc. of blood from the liver liberated 84 cc. of oxygen from hydrogen peroxide while 0.5 cc. of blood from the portal and jugular veins liberated 64 and 66 cc. respectively. After the introduction of the glycocoll the catalase of the blood of the liver, portal and jugular veins was increased as is indicated by the increase in the amount of oxygen liberated from the hydrogen peroxide. The catalase of the blood of the liver was increased, particularly during the first fifteen minutes, more rapidly than that of the portal or jugular veins. This is taken to mean that the glycocoll was stimulating the liver to an increased amount of catalase. That the liver is continuously replenishing the blood and hence the tissues with catalase is shown by the fact that the blood of the liver is always richer in catalase by 15 to 20 per cent than the blood from any other part of the body. The fact also, as shown in a previous publication, that the introduction of a substance, such as ethyl alcohol, which normally produces a great increase in catalase, produced a very small or no in-

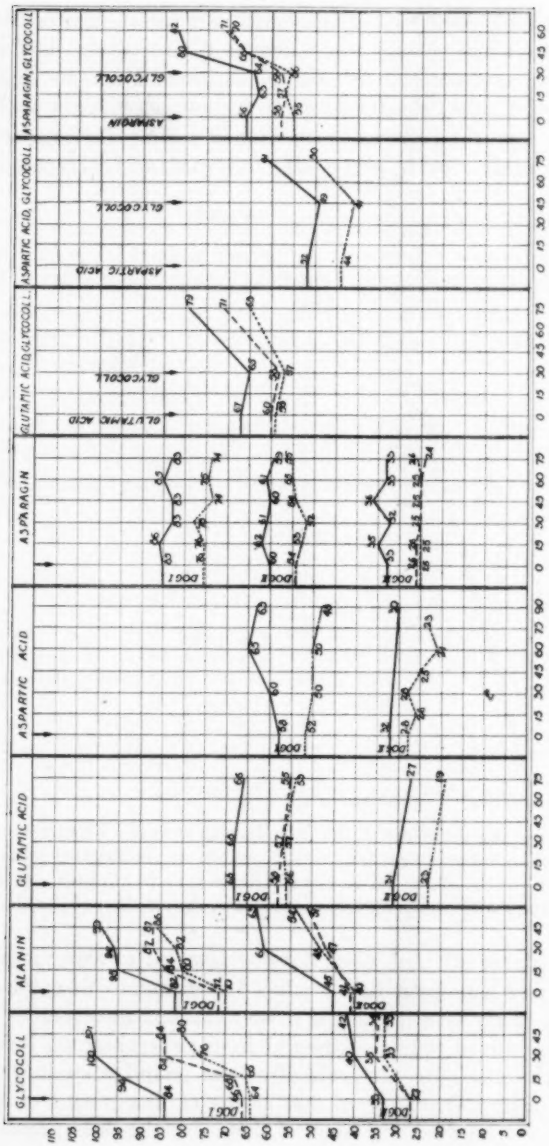

Fig. 1. Curves showing the effect on the production of catalase of the introduction into the intestines of glycocoll, alanin, glutamic acid, aspartic acid, asparagin, glutamic acid followed by glycocoll, aspartic acid followed by glycocoll, and asparagin followed by glycocoll. Continuous line curves show amount of catalase in the blood of the liver; discontinuous line curves show amount of catalase in the blood of the portal vein; dotted line curves show amount of catalase in the blood of the jugular. The figures along the abscissa indicate time in minutes and those along the ordinate, amounts of catalase measured in cubic centimeters of oxygen.

crease after the liver was cut out of the circulation by an Eck fistula and by ligating the hepatic artery was taken to mean that the liver is the organ principally responsible for the production of catalase. It may be seen that the alanin also produced an increase in catalase while the glutamic and aspartic acids and asparagin did not. It may also be seen under (glutamic acid, glycocoll), (aspartic acid, glycocoll)

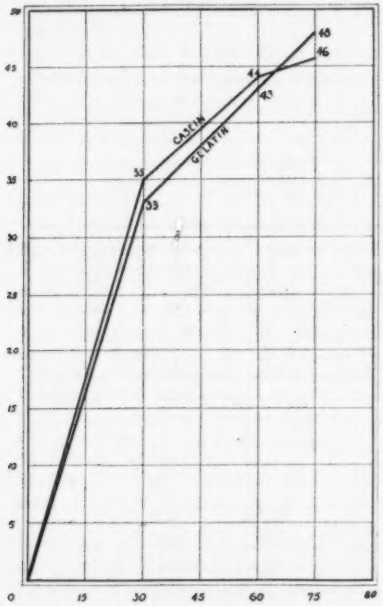

Fig. 2. Curves showing the effect of the introduction into the intestines of a pancreatic digest of an adequate protein, casein, and of an inadequate protein, gelatin. The figures (0–80) along the abscissa indicate time in minutes, and the figures (0–50) along the ordinate, percentage increase in the catalase of the blood.

and (asparagin, glycocoll) that after the introduction of aspartic and glutamic acids and asparagin had produced no increase in catalase, the introduction of glycocoll produced a very marked increase.

In figure 2 the curves were constructed from data obtained before as well as at fifteen-minute intervals after the introduction into the intestines of a pancreatic digest of 300 grams of casein and of 300 grams of gelatin. The digest of each of these proteins was prepared

and introduced as already described. It will be seen that the introduction of the digests of the adequate protein, casein, as well as of the inadequate protein, gelatin, increased the catalase of the blood very extensively, in keeping with the fact that the ingestion of these proteins increases oxidation.

SUMMARY

The ingestion of amino-acids such as glycocoll and alanin produces an increase in catalase with resulting increase in oxidation, by stimulating the digestive glands, particularly the liver, to an increased output of this enzyme; while the ingestion of amino-acids such as glutamic and aspartic produces no increase in catalase, in keeping with the fact that they cause no increase in oxidation.

The introduction of the digest of the adequate protein, casein, as well as the digest of the inadequate protein, gelatin, produces an increase in the catalase of the blood, in keeping with the fact that the ingestion of these proteins brings about an increase in oxidation.

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REGENERATION OF BLOOD SERUM PROTEINS

I. Influence of Fasting upon Curve of Protein Regeneration Following Plasma Depletion

WM. J. KERR,[1] S. H. HURWITZ AND G. H. WHIPPLE

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

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The experiments tabulated below were performed to obtain information concerning the regeneration and source of blood proteins, their fluctuation under abnormal conditions and their function in the body economy. The experimental evidence is sufficient to show that the regeneration of blood serum proteins is quite slow, in fact a matter of several days, following a marked depletion of blood proteins. This is in striking contrast to the rapid regeneration of fibrinogen (one of the blood plasma proteins), which has been studied previously by Whipple (1) and Goodpasture (2). Regeneration of the blood serum proteins can take place during starvation, although not as rapidly as on a full diet, but this shows that the body can manufacture these proteins at the expense of other body proteins or perhaps by conserving the amino acids which result from the protein katabolism of starvation.

It is not necessary to review the rather extensive literature on this subject as excellent reviews by Morawitz (3) in 1909 and Rowe (4) in 1916 are available. It is to be noted that all the earlier workers failed to demonstrate any constant fluctuations in the blood proteins during periods of fasting or after heavy feeding. Some observers noted a relative increase in globulins at the expense of albumins after long periods of fasting and others deny that these fluctuations are in excess of those noted in normal animals under the usual experimental conditions. We can agree with the general opinion that the blood serum proteins show but minimal fluctuations during periods of fasting or after heavy feeding and such fluctuations may be observed in normal

[1] Wm. J. Kerr, Sheldon Traveling Fellow in Research Medicine, 1916–1917, Harvard University, Cambridge, Massachusetts.

animals (compare table 1). Our observations go to show that the amounts of blood proteins in dogs may vary more in different animals than in the same animal. In other words, a given dog has his normal protein level which shows only slight fluctuations, but this level may differ considerably from the level of another normal dog.

The earlier workers employed methods for the estimation of blood proteins which required large amounts of blood and at once introduced the factor of hemorrhage if many observations were to be recorded on the same animal within a short period of time. The labor necessary to isolate these various proteins was great and precluded any large series of observations by any single worker. We felt that it was very important to make frequent observations during any given experiment in order to get any clear understanding of the curve of blood protein regeneration uninfluenced by great loss of blood.

The refractometric method of Robertson (5) for the estimation of blood protein lends itself admirably to such work and made possible these experiments. It is of interest to note that our figures are in accord with those of Morawitz (6), who used the older methods of precipitation and analysis but very similar methods to deplete the blood proteins. This indicates that the figures obtained by the refractometer corresponded to those obtained by the other methods. We have recorded elsewhere (7) the values of blood proteins observed in normal dogs and a detailed description of the methods.

METHODS

Dogs were used for all these experiments and all remarks unless otherwise stated refer to dogs and their blood serum proteins. All operative procedures were done under complete surgical ether anaesthesia. So far as we are aware, Morawitz was the first worker to employ the method of *plasma depletion* as used in these experiments. The method is very simple and consists of bleeding the animal from the artery and introducing into the vein red corpuscles washed and suspended in saline solution, Locke's solution or some modification of a protein-free solution. The red cell Locke's solution mixture may be given during the arterial bleeding or after the bleeding. Morawitz used the former method. This method was used by Abel, Rowntree and Turner (8) and called "Plasmapharesis." We wish to use the term to indicate a withdrawal of whole blood and replacement of washed red corpuscles suspended in Locke's solution or some modification of

this medium. However this procedure may be used it effects a dilution or depletion of the plasma and removes plasma proteins in large amounts. The experiment can be repeated frequently during the day and the blood proteins can be reduced to a low level without any anemia or fall in the volume of red blood cells. If these exchanges are done with care and proper regard for clinical evidences of shock, the plasma depletion can be carried from 6 per cent protein to 2 per cent and even as low as 1 per cent serum protein. If the exchanges are too rapid or too large in amount the dog will go into a condition of profound shock which will be discussed in detail in a future publication.

In our first experiments we had some trouble with subsequent infection at the site of operation in the neck or groin where the vessels were exposed, but improved technique did away with this factor and the wounds healed in two to three days with a minimum reaction. In these experiments the blood was drawn into clean glass containers into oxalate or citrate and centrifuged to separate the plasma. The red cells were washed at least twice to remove all proteins and suspended in Locke's solution or Locke's solution containing 3 to 4 per cent gum acacia made up to the original volume. These collections and washings were not done with complete aseptic precautions but some later experiments done with every aseptic caution gave identical results. In some experiments we used washed red corpuscles obtained from another normal dog, but in other experiments (tables 4, 5 and 6) the corpuscles were obtained from the same dog on the day before the complete experiment was carried out. No differences in experimental result can be attributed to these differences in red blood corpuscles.

The blood proteins were estimated by the refractometric method of Robertson (5). The greater part of the readings was made by the same individual and with but one exception the readings given in any one table were all made by the same individual to minimize fluctuations which may be due to personal factors.

Metabolism experiments were done in the usual cages which make possible an accurate collection of urine. The dogs were catheterized at the end of every 24-hour period, the bladder washed out, the animal weighed and given a uniform amount of water by stomach tube—usually 400 cc. The urinary collections and cage washings were made up to a unit volume and analyses of nitrogen by the Kjeldahl method done in duplicate.

The estimated blood volumes were calculated as 7.5 per cent of the body weight. Experiments soon to be published will indicate that this

figure is below the actual blood volume figure which will average about 10 per cent according to the accurate determinations in the normal dog made possible by an improved dye method for blood volume estimation. The figures for total blood volume per cent exchange as given in the tables are actually about 30 per cent too high. We hope to report other experiments in which this factor is accurately controlled.

EXPERIMENTAL OBSERVATIONS

All the results are given in the form of tables and unless otherwise stated the dogs were in normal condition during the entire experiment. It will be noted that several series of observations (for example, tables 4 and 5) were made upon the same animal under the same or different experimental conditions. This gives additional data concerning the normal fluctuations of plasma proteins during varying intervals of time in the same animal. Table 1 is given here to furnish data of a

TABLE 1

Serum albumin, globulin and total protein after feeding and after fasting

DATE	FEEDING OR FASTING	TOTAL PROTEIN	TOTAL ALBUMIN	TOTAL GLOBULIN	ALBUMIN	GLOBULIN	NON-PROTEIN CONSTITUENTS	PROTEIN QUOTIENT
					per cent	*per cent*	*per cent*	
December 30	Starved 3 days (?)	7.0	4.8	2.2	70	30	1.8	2.3
January 3	3 days of mixed diet 3 hours after meal	6.6	4.1	2.5	62	38	1.7	1.6
January 4	Bled 24 hours after feeding	6.4	4.5	1.9	70	30	1.6	2.3
January 10	6 days of fast	6.5	4.2	2.3	64	36	1.6	1.7
January 13	2 hours after feeding	6.1	4.0	2.1	65	35	1.6	1.9
January 27	After a feeding	5.4	3.6	1.8	66	34	2.0	1.9

characteristic experiment which shows the negative result of fasting and heavy feeding upon the blood serum proteins of a normal dog. This is in harmony with the work of other experimenters who used different methods. "Protein quotient" in table 1 is a figure obtained by dividing the albumin per cent by the globulin per cent and does not vary very greatly in a normal dog. With increase in the globulin this protein quotient approaches one and there may be inversion of the

normal ratio giving a protein quotient of 0.5 or even less. Such inversion is often present in infections and intoxications as pointed out in detail in other publications from this laboratory (7 and 9). The "protein quotient" is not figured in the other charts as we do not wish to emphasize this figure in these experiments.

Tables 2 and 3 give the results of two experiments which are good examples of others similarly performed. Table 2 illustrates a moder-

TABLE 2

Dog 16–4. Regeneration of serum proteins. Fasting

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total protein	Total albumin	Total globulin	Non-protein constituents		
1916	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
May 4	5.5	4.0	1.5	2.2	22.50	Fasting begun
May 6	5.4	3.5	1.9	2.0		
May 10						
Specimen 1	5.1	2.6	2.5	3.0	20.13	*Plasmapharesis:* Total blood withdrawn 505 cc. R. B. C. suspension injected 520 cc. done in 3 bleedings. Total proteins reduced 40 per cent
Specimen 2	4.3	2.1	2.2	2.7		
Specimen 3	4.2	2.4	1.8	2.4		
Specimen 4	3.1	1.1	2.0	2.5		
May 11	3.6	1.9	1.7	2.5	19.13	Animal active. Wound in good condition
May 12	4.0	2.0	2.0	2.4	19.00	
May 13	3.1	1.5	1.6	2.4		
May 14	4.3	3.2	1.1	2.8	18.25	
May 15	4.3	2.0	2.3	2.6		
May 16	4.5	3.0	1.5	2.6	17.50	
May 17	3.8	1.0	2.8	2.6	17.13	
May 18	5.0	2.6	2.4	2.2	17.00	
May 19	5.0	2.5	2.5	2.0	16.00	
May 20	5.2	2.9	2.3	2.1	16.50	

ate depletion of serum proteins to only 60 per cent of normal. The body is able to regenerate to the normal serum protein level only after eight days, with zero food intake. It will be seen that in the days following the plasmapharesis the globulins tend to heap up faster than the albumins. This may be due to some infection during the experiment or more probably to the shock of the experiment as shown in later tables. It may be evidence that the serum globulins are regenerated

faster than the albumins or that during this forced regeneration the precipitation values of these proteins may vary from normal.

Table 3 gives the results of a slightly different experiment. At the beginning of the experiment a large amount of Locke's solution was introduced and this was followed at once by the usual plasmapharesis

TABLE 3

Dog 16–113. Hydraemic plethora. Plasmapharesis. Regeneration of serum proteins. Fasting

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total protein	Total albumin	Total globulin	Non-protein constituents		
1917	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
May 15	4.9	3.4	1.5	2.3	16.75	Bled 170 cc. into 20 cc. 10 per cent sodium citrate solution
May 16					16.25	Hydraemic plethora. 1000 cc. Locke's solution introduced into femoral vein before plasmapharesis
Specimen 1	2.8	1.7	1.1	1.7		
Specimen 2	2.5	1.6	0.9	2.0		
Specimen 3	1.8	0.9	0.9	2.2		*Plasmapharesis:* Estimated blood volume 555 cc. Bled 1070 cc. in 4 bleedings of 150–400 cc. each. Total 192 per cent of blood volume. Reinjected 1920 cc.
Specimen 4	1.6	0.8	0.8	2.3		
Specimen 5	1.7	0.9	0.8	2.5		
May 17	2.5	1.2	1.3	2.1	16.25	Water ad lib.
May 18	2.9	1.3	1.6	2.4	15.75	Wound O.K.
May 19	3.2	1.8	1.4	2.3	15.50	
May 21	3.6	1.9	1.7	2.2	14.63	Slight hemolysis
May 22	3.7	1.7	2.0	2.0	14.25	
May 23	3.6	1.9	1.7	2.1	14.00	
May 24	3.9	1.9	2.0	1.9	13.63	
May 25	3.8	1.9	1.9	2.0	13.25	On bread and milk diet after bleeding
May 26	3.6	2.0	1.6	1.7	13.63	Bread and milk diet
May 28	3.9	2.1	1.8	1.8	13.25	Bread and milk diet
May 30	3.9	2.1	1.8	1.8	13.63	Meat diet after bleeding
May 31	4.0	1.6	2.4	1.9		Slight turbidity of serum. Meat diet
June 1	4.1	2.3	1.8	2.0	13.50	Meat diet
June 2	4.1	2.1	2.0	1.8	14.00	Meat diet
June 4	4.8	2.9	1.9	1.8	13.63	Experiment discontinued

during which 920 cc. of red blood cell suspension were injected and bleedings of 1070 cc. were removed. This procedure removes more blood protein but also intensifies the shock and the chances of a fatal result are greater. Removal of 192 per cent of the estimated blood volume lowered the blood proteins to 1.7 per cent The curve of regeneration is quite slow during nine days of fasting and was not rapid even on a meat diet. Thus table 3 illustrates the difficulty with which the body regenerates serum protein when the circulating protein is depleted and probably the reserve supply called out. During the first week there is a tendency for the globulins to increase more rapidly than the albumins.

The three tables below (tables 4, 5 and 6) are very similar and give nteresting data concerning the basal nitrogen metabolism. Following the plasmapharesis there is a definite rise of nitrogen elimination in the urine—often one or two grams of nitrogen above the normal base line to be noted in each of the two days following the plasmapharesis. The curve of nitrogen elimination resembles exactly that reported by Whipple and Cook (10) following the intravenous injection of proteose material. We wish to assume that in both cases we are recording a *definite injury to body protein*, in one case due to the sudden dilution of the plasma and in the other case due to the action of toxic proteose on the body protein.

Furthermore, there is no evidence of any increased protein breakdown during the period of prolonged effort on the part of the body to build up the blood serum proteins. There is the period of two to three days or longer following the plasmapharesis in which the nitrogen elimination is much above normal; then follows a period of normal nitrogen elimination for a week or more while the body is still making up the serum protein deficit. During the last period there is no evidence that the body is breaking down protein to furnish the material to construct the necessary serum proteins. The protein breakdown is that normally observed in the fasting dog. Some observers may wish to explain the primary rise in urinary nitrogen after plasmapharesis as due to an emergency reaction to supply the urgently needed serum proteins. We believe the evidence is more in favor of the explanation that the rise in basal nitrogen elimination is due to actual injury of body protein associated with the shock of the plasma dilution. Table 6 gives an excellent example of acute and almost fatal shock following a small but rapid plasmapharesis. An exchange of 50 per cent of the estimated blood volume caused severe clinical shock with low blood pressure and

temperature, vomiting and diarrhoea. Dog was in poor condition when removed to his cage. The recovery the next day was perfect and the rise in basal nitrogen is not marked.

TABLE 4

Regeneration of serum proteins. Metabolism

DATE	SERUM PROTEINS				NITROGEN ELIMINATION	WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents			
1917	*per cent*	*per cent*	*per cent*	*per cent*	*grams*	*pounds*	
February 19.....	5.2	3.6	1.6	2.0	1.68	19.38	400 cc. water daily by stomach tube
February 20.....	4.9	3.5	1.4	2.2	1.68	19.06	Bled 150 cc. into sodium citrate solution
February 21.....					2.10	18.44	*Plasmapharesis:* Estimated blood volume 630 cc. Bled 740 cc. in 5 bleedings of from 75 to 150 cc. each = 120 per cent of blood volume. Reinjected 845 cc. of Locke's acacia solution
Specimen 1....	4.7	3.5	1.2	2.4			
Specimen 2....	3.9	3.1	0.8	2.2			
Specimen 3....	3.1	1.8	1.3	2.4			
Specimen 4....	3.1	2.0	1.1	2.5			
Specimen 5....	2.6	2.0	0.6	2.9			
Specimen 6....	2.2	1.8	0.4	3.1			
February 22.....				3.1	3.89	18.44	Hemolysis
February 23.....	2.7	1.2	1.5	3.7	4.45	17.63	Hemolysis
February 24.....	3.1	1.1	2.0	3.3	3.14	17.13	400 cc. water by stomach tube
February 25.....					3.58	17.00	
February 26.....	3.5	2.0	1.5	2.9	3.00	16.63	
February 27.....	4.5	2.8	1.7	2.7	2.44	16.44	
February 28.....	4.1	2.3	1.8	2.5	2.13	16.19	
March 1.....	3.9	2.2	1.7	2.6	1.82	15.88	
March 2.....					1.74	15.56	75 gm. sugar in 400 cc. water by gavage
March 3.....	4.3	2.6	1.7	2.2	2.02	15.38	75 gm. sugar in 400 cc. water
March 4.....					1.57	14.88	
March 5.....	5.0	2.8	2.2	2.4	1.40	14.63	Small amount lean meat
March 6.....	4.9	3.1	1.8	2.5			Liberal meat diet
March 8.....	5.9	3.3	2.6	2.3		16.00	Liberal meat diet

Tables 4 and 5 give the results of similar experiments on the same dog at different times and illustrate beautifully the slow protein regeneration which follows the depletion of a plasmapharesis. The normal

TABLE 5

Dog 17–140. Regeneration of serum proteins. Metabolism

DATE	SERUM PROTEINS				NITROGEN ELIMINATION	WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents			
1917	*per cent*	*per cent*	*per cent*	*per cent*	*grams*	*pounds*	
April 24	4.9	3.5	1.4	2.4	2.41	20.25	Bled 220 cc. into citrate solution
April 25					2.38	19.19	*Plasmapharesis:* Estimated blood volume 690 cc. Bled 700 cc. in 4 bleedings of 150 to 180 cc. each. Total 101 per cent of blood volume. Reinjected 860 cc. in Locke's acacia solution
Specimen 1	4.5	3.0	1.5	2.1			
Specimen 2	4.3	3.1	1.2	2.0			
Specimen 3	3.7	2.6	1.1	2.1			
Specimen 4	3.4	2.5	0.9	2.2			
Specimen 5	2.3	1.7	0.6	2.8			
April 26	2.9	1.8	1.1	2.7	3.50	19.13	Slight infection of wound
April 27	3.2	1.7	1.5	2.8	3.30	18.25	400 cc. water daily by stomach tube
April 28	3.7	2.3	1.4	2.2		18.06	
April 29	3.0	1.3	1.7	1.3	2.38	17.88	Marked hemolysis
April 30	4.3	2.4	1.9	2.1	2.44	17.63	Moderate hemolysis
May 1	4.2	2.2	2.0	2.1	2.44	17.63	Moderate hemolysis
May 2					2.04	17.13	
May 3	4.2	1.8	2.4	2.3	2.38	16.81	
May 4	4.1	2.2	1.9	1.8	1.96	16.56	
May 5	4.0	1.9	2.1	2.0	1.93	16.31	
May 6					1.76	15.94	
May 7	4.1	2.4	1.7	1.8	1.99	15.75	Slight hemolysis
May 8	4.2	2.9	1.3	1.7	1.96	15.44	Moderate hemolysis
May 9	4.1	2.5	1.6	1.9	1.88	15.13	
May 10	4.6	2.7	1.9	1.8	2.07	15.00	Moderate hemolysis
May 11	4.7	2.7	2.0	1.9	2.04	14.81	Moderate hemolysis. 50 gm. cane sugar in water by gavage
May 12	4.4	2.4	2.0	2.0	1.62	14.25	50 gm. cane sugar and 25 gm. glucose in water by gavage
May 13	4.5	2.7	1.8	2.2			50 gm. cane sugar and 25 gm. glucose in water by gavage

TABLE 5—*Continued*

DATE	SERUM PROTEINS				NITROGEN ELIMINATION	WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents			
1917	*per cent*	*per cent*	*per cent*	*per cent*	*grams*	*pounds*	
May 14.....	4.4	2.7	1.7	2.4	1.48	14.13	50 gm. cane sugar and 25 gm. glucose in water by gavage
May 15.....	4.7	3.0	1.7	2.1	1.65	14.00	Metabolism discontinued. On bread and milk diet after bleeding
May 16.....	4.4	2.7	1.7	2.4		14.50	Bread and milk diet
May 17.....	4.3	2.6	1.7	2.1		15.25	Bread and milk diet
May 18.....	4.4	2.9	1.5	2.0		15.50	Bread and milk diet
May 19.....	4.3	3.1	1.2	2.0		15.88	Bread and milk diet
May 21.....	4.6	2.7	1.9	2.1		15.75	On meat diet after bleeding
May 22.....	5.0	3.1	1.9	2.2		15.50	Meat diet
May 23.....	5.0	3.2	1.8	2.2		16.06	Meat diet. Slight hemolysis
May 24.....	4.8	2.4	2.4	2.9		17.13	Moderate lipemia. Experiment discontinued

healthy fasting dog can regenerate serum proteins only with difficulty and complete repair from a depletion of 40 to 50 per cent takes many days—usually ten or more. In many similar experiments we have no record of complete regeneration to the normal level while the dog was fasting. When the depletion is not as great the regeneration may take the serum proteins practically back to normal in about ten days, with no food intake. Table 4 shows in an extreme degree the change which may appear in the non-protein fraction by this refraction metric method. During a plasmapharesis there is usually a rise in the non-protein fraction and this may appear on several days following the plasma depletion. The increase in this experiment is almost 50 per cent above normal (table 4) and the non-protein fraction remains high on the three succeeding days. There is no similar change in the non-protein nitrogen as determined by the usual methods—for example, by trochloracetic acid precipitation of serum proteins and Kjeldahl determinations of nitrogen in the filtrate. During a long plasmapharesis the urea and non-protein nitrogen of the blood plasma determined by

TABLE 6

Dog 17–130. Regeneration of serum proteins. Metabolism

DATE	SERUM PROTEINS				NITROGEN ELIMINATION	WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents			
1917	*per cent*	*per cent*	*per cent*	*per cent*	*grams*	*pounds*	
March 26	5.8	3.1	2.7	1.9	2.05	13.94	400 cc. water daily by stomach tube
March 27	5.6	3.1	2.5	1.7	2.13	13.75	Bled 150 cc. in 15 cc. 10 per cent sodium citrate solution
March 28					2.18	13.19	*Plasmapharesis:* Estimated blood volume 450 cc. Bled 230 cc. in 2 bleedings = 50 per cent blood volume. Reinjected 450 cc. in Locke's acacia. Animal in shock. Diarrhoea. Returned to cage in poor condition
Specimen 1	5.3	3.0	2.3	1.5			
Specimen 2	4.4	2.6	1.8	1.7			
Specimen 3	2.9	1.2	1.7	2.1			
March 29	3.6	1.7	1.9	2.1	2.44	13.13	Good condition
March 30	3.9	2.0	1.9	2.2	2.63	12.63	400 cc. water daily by stomach tube
March 31	3.9	1.9	2.0	1.9	2.04	12.56	Wound O.K.
April 1					1.96	12.19	
April 2	4.2	2.1	2.1	1.4	1.88	12.19	
April 3	4.1	1.8	2.3	1.8	1.85	11.94	
April 4	4.2	1.7	2.5	1.9	1.74	11.81	
April 5	4.0	1.5	2.5	1.7	1.85	11.69	
April 6	3.6	0.9	2.7	1.8	1.97	11.19	
April 7	4.4	1.8	2.6	1.7	1.65	11.06	Slight hemolysis of serum
April 8					1.57	10.81	
April 9	4.4	1.4	3.0	2.0	1.60	10.88	Animal weak. Bread and milk diet. Small amount
April 10	4.6	2.6	2.0	2.0	1.51	10.50	Feedings increased
April 11	4.1	1.3	2.8	2.2		11.88	Extra diet
April 13	4.4	2.1	2.3	3.0		12.31	Lipaemic serum
April 14	4.9	2.2	2.7	2.4			Lipaemic serum
April 16	5.2	2.6	2.6	2.0		13.25	
April 17	4.9	2.4	2.5	1.9			
April 18	5.0	3.2	1.8	1.7			Experiment discontinued

any of the clinical methods will show a slight rise above normal—even at times 50 per cent above normal, but this change is very transient and returns to normal in a few hours.

The non-protein fraction as given in the charts represents a great variety of substances in the serum not precipitated by acetic acid—the salts, sugar, urea, etc. What substance explains the increase in the non-protein fraction under these conditions we do not venture to state. There may even be a change in the precipitation value of the proteins. Work is in progress which may throw some light on this point.

DISCUSSION

Many efforts have been made to show some definite relationship between the blood proteins, tissue proteins and food proteins. It is a natural enough assumption that the circulating blood proteins play an important part in nutrition of body cells, but all efforts to prove this point have failed and the more recent work on the amino acids makes it clear that plasma proteins do not necessarily represent a transition stage between food protein and body tissue protein. It is known that the blood proteins show no definite change in hunger or after heavy protein feeding, but we are familiar with other substances in the blood which maintain a very constant level in health (for example, glucose) where a rapid transfer is known to take place between the food and body tissues through the blood. So the constant level of blood proteins in hunger or after feeding gives no proof that the blood proteins are not concerned in the transfer of proteins from the food to the body tissues. But when by experiment we reduce the blood proteins to a low level (to 33 per cent or even to 20 per cent of normal) and follow the slow regeneration to normal in five to ten days on a meat diet we are fairly certain that these blood serum proteins cannot be actively concerned as *transition products* of protein metabolism standing between food proteins and body tissue proteins; otherwise the return to normal would be expected to take place within twenty-four hours or less. We are convinced that these experiments give sufficient proof that the blood serum proteins are not directly concerned in the protein metabolism as intermediary products of exchange between food protein and body tissue protein. We hope to submit evidence to show that these blood serum proteins serve a very important function in maintaining the normal equilibrium of the parenchyma cells.

When the serum proteins have been depleted to a low level by means of repeated plasmapharesis the dog will show a slow regeneration of these plasma proteins. On starvation this curve may not rise to normal in ten to fourteen days yet the nitrogen elimination in the urine may be normal after the first few days. There will be no variation from normal in the blood urea and non-protein nitrogen after the evidences of clinical shock have vanished. Yet we cannot say with conviction that the body does or does not break down any excess body protein to form the serum proteins which surely are formed during this period of zero protein intake. We may assume that the body conserves certain amino acids and builds them into the necessary serum proteins but there is no evidence of nitrogen retention (tables 4, 5 and 6). It must be admitted that the body may break down cell protein and recast it into serum protein without changing the basal nitrogen elimination. These several possibilities are to be considered and at this time we cannot emphasize one to the exclusion of another.

SUMMARY

Serum proteins may be reduced to a low level in the normal dog by means of repeated bleedings and simultaneous injections of washed blood cells suspended in some protein-free medium (e.g., Locke's solution).

Regeneration of the serum proteins may be followed after this type of plasma depletion (plasmapharesis). This regeneration is a slow and difficult matter occupying many days—often seven to fourteen days when the original depletion removed 50 per cent or more of the total serum protein.

This depletion presumably is the maximal stimulus for body production of serum protein, yet there is a marked delay in regeneration. It seems that the body can produce serum proteins only in small amounts even in an emergency.

Serum proteins can be regenerated when there is no protein intake in the food, therefore the serum proteins must be formed from the body proteins under such circumstances.

The basal nitrogen metabolism shows no constant variation under these experimental conditions, with the exception of a primary rise in nitrogen elimination which occurs in the few days following the shock of the plasmapharesis.

There is a tendency for the curve of globulin regeneration to rise

faster than that of the serum albumin. This may be due to the initial shock of the plasmapharesis or to some infection and slight wound reaction. It may possibly be due to more rapid production of serum globulin.

We have no evidence of any constant fluctuation in serum proteins in association with periods of fasting or heavy protein feeding. The changes observed come within the limits observed in normal dogs under uniform conditions.

These experiments give no evidence that the serum proteins may be in any way concerned as intermediary products between food protein and body tissue or parenchyma protein.

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REGENERATION OF BLOOD SERUM PROTEIN

II. Influence of Diet upon Curve of Protein Regeneration Following Plasma Depletion

WM. J. KERR,[1] S. H. HURWITZ AND G. H. WHIPPLE

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

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The experiments tabulated below supplement those in the preceding paper and must be compared with them in any analysis of results. The experiments were performed under identical conditions described previously and the figures may be compared without any allowance being necessary to cover individual variations in technique. The conditions of the experiments and methods have been described in the preceding communication.

It is evident that the curve of serum protein regeneration rises more promptly to normal when the dog is on a full diet as compared with a similar experiment upon a fasting animal. A meat diet seems to be most efficient in effecting a rapid regeneration of serum protein. Even under the most favorable conditions of diet it requires from five to eight days to regenerate the serum proteins to normal when the depletion has been carried to 50 per cent or 33 per cent of normal. This shows that the body cannot form serum protein with much more speed than it can form new cells in parenchymatous organs (e.g., liver cells).

EXPERIMENTAL OBSERVATIONS

Tables 10 and 11 show very clearly the sharp rise in the regeneration curve of serum proteins which is associated with a *meat diet*. In the first experiment the depletion of serum protein was not extreme—a reduction to 2.9 per cent—and the regeneration may be considered complete in five days, although the abnormally high figure of the first

[1] Wm. J. Kerr, Sheldon Traveling Fellow in Research Medicine, 1916–1917, Harvard University, Cambridge, Massachusetts.

reading is not attained. There was no notable increase in the globulin fraction.

The second experiment shows a great depletion of the serum proteins effected by eight bleedings amounting to 157 per cent of the estimated blood volume. The rise in the non-protein fraction is well illustrated. The curve of regeneration is rapid and complete in eight days in spite of the initial low level.

TABLE 10

Dog 16–129. Regeneration of serum proteins. Meat diet

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1916	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
March 15						
Specimen 1...	6.4	5.0	1.4	2.2	14.00	*Plasmapharesis:* Estimated blood volume 480 cc. Four bleedings each of 135 cc. Total withdrawn 540 cc. Reinjected 600 cc. Total protein reduced 55 per cent
Specimen 2...	5.4	4.1	1.3	2.1		
Specimen 3...	4.7	4.0	0.7	2.2		
Specimen 4...	3.5	2.5	1.0	2.4		
Specimen 5...	2.9	1.8	1.1	2.7	13.75	
March 16.....	3.6	2.3	1.3	2.0	14.00	Dog active. Wound in good condition
March 17.....	4.2	2.5	1.7	1.9	13.50	Meat diet
March 18.....	4.4	2.8	1.6	1.8	14.50	
March 19.....	4.5	2.1	2.4	2.0	15.00	
March 20.....	5.0	3.0	2.0	1.9		
March 22.....	5.1	3.0	2.1	2.2	15.50	
March 29.....	5.4	3.8	1.6	2.2	17.00	

Tables 12 and 13 show the curve of serum protein regeneration on a "mixed diet." By this term we mean a diet of table scraps including bones, meat, bread scraps, potatoes, rice, etc., given in liberal amounts once per day. Such a diet of course is rich in a variety of proteins. Table 12 records the serum protein fluctuations in an experiment in which the plasma depletion was considerable and the shock very severe. Two exchanges were performed each of 500 cc. which was slightly more than the total estimated blood volume. Such large exchanges are always associated with shock and often with fatal shock.

This dog showed profound shock and had not recovered the next day when blood-stained feces were passed. The depletion of the blood plasma proteins is not as great as attained by a series of smaller bleedings but the figures are relatively low. In spite of the profound shock, the regeneration of total proteins during the first twenty-four hours amounts to a little more than 1 per cent. The regeneration may be

TABLE 11

Dog 16–140. Regeneration of serum proteins. Meat diet

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1917	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
February 26....	4.9	3.2	1.7	3.1	23.25	
February 27....	4.6	2.6	2.0	2.8		Bled 175 cc. into 20 cc. 10 per cent sodium citrate solution
February 28					23.00	*Plasmapharesis:* Estimated blood volume 787 cc. Bled 1250 cc. Seven bleedings from 140 to 200 cc. each. Total 157 per cent of blood volume. Reinjected 1325 cc. in Locke-acacia solution
Specimen 1...	4.8	3.2	1.6	2.6		
Specimen 2...	4.1	2.8	1.3	2.2		
Specimen 3...	2.6	1.8	0.8	3.3		
Specimen 4...	3.4	2.5	0.9	2.5		
Specimen 5...	2.5	1.8	0.7	3.3		
Specimen 6...	2.2	1.8	0.4	3.5		
Specimen 7...	1.8	1.3	0.5	3.6		
Specimen 8...	1.7	1.3	0.4	3.6		
March 1.....	2.5	1.2	1.3	3.3	22.25	Dog normal
March 2.....	3.2	1.5	1.7	3.2	22.50	
March 3.....	4.0	2.3	1.7	2.5		
March 5.....	4.2	2.3	1.9	2.1		
March 6.....	4.4	2.4	2.0	2.0	23.00	
March 8.....	5.0	2.6	2.4	2.0	22.50	

looked upon as complete in six days. There was practically no upset in the globulin-albumin ratio in spite of the shock and slight infection of the neck wound.

Table 13 shows an experiment in which the depletion was effected by smaller exchanges but a lower final figure was attained than in the preceding experiment (table 12). The non-protein fraction shows the rise usually noted. There was a minimum amount of shock and the dog

was in excellent condition at the end of the experiment. In spite of the great difference in the general condition of the two dogs (tables 12 and 13) the amount of serum protein regeneration during the first twenty-four hours following the plasmapharesis is identical. It will be noted that this figure is constant in most of the experiments regardless of shock or its absence or abundant food or fasting. It seems that

TABLE 12

Dog 16–106. Regeneration of serum proteins. Mixed diet

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1916	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
February 16....						*Plasmapharesis:* Estimated blood
Specimen 1...	6.0	5.4	0.6	2.0	14.00	volume 472 cc. Two bleedings, each of 500 cc. R. B. C.
Specimen 2...	3.1	1.3	1.8	2.8		for first injection obtained on
Specimen 3...	2.0	1.5	0.5	3.0		day previous from another animal and suspended in Locke-acacia solution
February 17....	3.2	2.0	1.2	2.5	13.75	Dog drowsy—passed some bloody urine and bloody stool. Does not eat. Temperature 38.7°
February 18....	3.5	2.9	0.6	2.8	14.25	Dog more active. Eating. Some suppuration of neck wound
February 19....	4.4	3.6	0.8	2.1		Mixed diet—dog normal
February 20....	4.5	2.9	1.6	2.2		
February 21....	4.9	2.0	2.9	1.9	14.50	
February 22....	5.5	3.8	1.7	1.8		
February 23....	4.8	2.6	2.2	1.8		
February 24....	5.4	3.3	2.1	1.5	15.00	
February 26....	5.1	3.4	1.7	2.0		

this must be an emergency reaction and represents the supreme effort on the part of the body.

Table 14 shows an experiment which is complicated by infection and suppuration. The globulins show a considerable rise at the expense of the albumins. These fluctuations occur while the curve of total protein regeneration follows a slow upward trend. There seems to be more delay in this experiment than usual in uncomplicated experiments

with mixed diet. We have other observations to indicate that a severe infection will tend to slow up the speed of regeneration. It is not certain whether this delay may not be explained at least in part by impaired appetite and digestion. The primary depletion was to an extremely low level as can be seen by comparison with other experiments. We find that 1 per cent is about the lowest figure obtainable

TABLE 13

Dog 17-69. Regeneration of serum proteins. Mixed diet

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1917	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
January 29, 30..	5.3	2.9	2.4	2.4	15.00	Bled 75 cc. into 7.5 cc. 10 per cent sodium citrate solution
January 31					15.00	*Plasmapharesis:* Estimated blood volume 510 cc. Five bleedings from 85 to 150 cc. each. Total 710 cc. Reinjected 885 cc. in Locke-acacia solution. Bleedings totaled 144 per cent of blood volume.
Specimen 1...	3.2	1.2	2.0	2.8		
Specimen 2...	2.7	1.8	0.9	3.3		
Specimen 3...	2.3	1.8	0.5	3.4		
Specimen 4...	1.8	1.0	0.8	3.4		
Specimen 5...	1.4	0.4	1.0	3.6	15.50	Returned to cage in good condition
February 1....	2.5	1.2	1.3	3.2	15.50	Animal active. Mixed diet
February 2....	3.7	1.2	2.5	2.5		Wound in neck slightly septic
February 3....	3.6	1.7	1.9	2.9		Neck septic
February 4....		2.1		3.8		Lipemic serum
February 5....	3.7	2.4	1.3	2.7		Wound healing
February 6....	4.4	1.8	2.6	2.4		
February 7....	4.1	2.6	1.5	2.4		Wound O.K.
February 8....	5.0	3.5	1.5	2.2	14.50	
February 12....	5.1	4.4	0.7	2.4	14.25	Experiment discontinued

by plasma depletion. It seems that 1 per cent serum protein is an absolute minimum below which the body cells cannot survive. Many dogs will show fatal shock before the serum proteins are reduced even to 2 per cent and there is much individual variation in resistance to the shock of plasmapharesis. This dog required twelve days to regenerate approximately to normal. The complicating amputation was due to a

thrombus probably the result of infection and trauma. The animal was sacrificed at the end of the experiment and autopsy revealed no abnormalities except those described.

Table 15 shows an uncomplicated experiment with serum protein regeneration following a moderate depletion, the dog being on a liberal bread and milk diet. There was no severe shock and no infection,

TABLE 14

Dog 16–127. Regeneration of serum proteins. Mixed diet

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1916	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
March 8.....						
Specimen 1...	6.4	6.0	0.4	2.1	15.50	*Plasmapharesis:* Estimated blood volume 525 cc. Five bleedings 135 cc. each. Total amount of blood removed 675 cc. Reinjected 750 cc. Total protein reduced 83 per cent
Specimen 2...	3.9	3.2	0.7	2.4		
Specimen 3...	2.9	2.4	0.5	3.0		
Specimen 4...	2.3	2.1	0.2	3.4		
Specimen 5...	1.4	1.1	0.3	4.1		
Specimen 6...	1.1	1.1	0	4.3	15.88	
March 9.....	2.4	1.5	0.9	3.0		Animal drowsy; oedema of left foreleg
March 10.....	2.6	1.3	1.3	2.8		Eats little
March 11.....	3.0	1.3	1.7	3.0	15.00	
March 12.....	3.3	1.6	1.7	2.5		Dog ill. Temperature 40.0°. Moist gangrene of left foreleg
March 13.....	3.8	2.6	1.2	2.0	14.50	Amputation of left foreleg
March 14.....	3.3	1.0	2.3	2.1		
March 15.....	3.8	1.5	2.3	2.0	13.50	Dog active. Eats well
March 16.....	3.6	0.6	3.0	2.0	14.00	Abscess on back
March 17.....	4.2	2.4	1.8	1.8		Abscess incised
March 20.....	5.4	1.5	3.9	1.8	14.00	Dog active. Eats. Abscess wound granulating

yet we note on several days a definite increase of globulins at the expense of the albumins. The curve of total protein regeneration is uniform but the speed of regeneration is not as great as upon a meat diet. This table also gives figures to indicate a moderate rise in the blood non-protein nitrogen during the various stages of the plasma depletion. Such changes are much more marked when there is severe

clinical shock which is associated with considerable breakdown of body protein (see preceding paper).

TABLE 15

Dog 16–124. Regeneration of serum proteins. Bread and milk diet

DATE	SERUM PROTEIN				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1916	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
March 22.....						
Specimen 1...	6.1	3.6	2.5	2.7	16.00	*Plasmapharesis:* Total withdrawn 600 cc. Reinjected 600 cc.
Specimen 2...	4.0	2.7	1.3	2.8		Blood N. P. Nitrogen 37 mgm., urea 28 mgm.
Specimen 3...	3.1	1.6	1.5	2.9		Blood N. P. N. 38 mgm., urea 28 mgm.
Specimen 4...	2.6	2.2	0.4	3.0		Blood N. P. N. 46 mgm., urea 27 mgm.
March 23.....	3.7	2.5	1.2	2.5	16.00	Dog active. Wound in good condition
March 24.....	3.9	2.6	1.3	2.2		Bread and milk 400 grams
March 25.....	4.5	2.6	1.9	1.8	16.00	Bread and milk 100 grams
March 27.....	4.6	2.0	2.6	2.0	16.00	Bread and milk 500 grams
March 28.....	4.5	1.6	2.9	2.2	15.75	Very active
March 29.....	4.9	1.7	3.2	1.9	16.00	Bread and milk 500 grams
April 1.....	5.0	2.1	2.9	2.1	15.50	Bread and milk 450 grams
April 3.....	5.2	3.0	2.2	2.1	16.00	

DISCUSSION

It is of interest to recall the fact that the body regenerates its *cell protoplasm* with the same difficulty and delay following cell injury. This statement applies particularly to the parenchymatous organs, as the liver and kidney. Whipple and Sperry (1) have shown that the liver injured by chloroform will repair the damaged cell protoplasm within seven to ten days, depending upon the severity of the original cell necrosis. Mr. Davis in this laboratory is investigating the curve of the liver cell regeneration and finds certain points of similarity between the parenchyma regeneration and the blood serum protein

regeneration. It may well be that the protein for the parenchyma cell or for the blood plasma may require similar construction periods and building material. It may be that the blood protein construction may depend upon the activity of the cell protoplasm. It is possible that all protein material may be built up part way alike, to be differentiated in the last stage of the complete elaboration.

We wish to point out the fact that after the initial depletion of serum proteins the body can almost always regenerate 1 per cent total protein during the first twenty-four hours following the plasmapharesis. This figure is remarkably constant and does not seem to be influenced by diet or fasting. The amount of shock does not appear to influence it because it may be the same in two experiments one of which is associated with grave shock (table 12) and the other with a minimum amount of shock (table 13). We may perhaps look upon this as the maximum effort of the body to replace these essential serum proteins, an effort which surely depends upon the body protein as it is present in fasting experiments. It will be the same whether there is a marked breakdown of host protein as evidenced by great increase in urinary nitrogen or whether this tissue autolysis is minimal.

SUMMARY

Regeneration of serum proteins is more rapid and complete upon a meat or mixed diet as compared with fasting experiments see (fig. 1).

The same regeneration does not appear to be as rapid upon a bread and milk diet as upon a meat diet.

Following the plasma depletion there is an average regeneration of 1 per cent total serum proteins within the next twenty-four hours. This is a uniform emergency reaction on the part of the body and is independent of food, shock or infection. It probably represents the absolute maximum production under the greatest stimulus and perhaps represents a throwing in of emergency reserve material.

The body has the same difficulty in regenerating its serum proteins, as it does in the construction of cell protein—for example, the repair of liver protoplasm following a chloroform necrosis.

Regeneration of serum protein after a 50 per cent depletion on a meat diet may be effected in five to seven days.

Regeneration of liver cell protoplasm after a 50 per cent necrosis may be effected in five to seven days. This is an interesting parallel which may have some significance.

The body cannot tolerate a reduction of its total serum protein from an average of 5 per cent to the low level of 1 per cent. This is practically always associated with fatal shock which may supervene long before the minimum figure of 1 per cent has been attained.

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REGENERATION OF BLOOD SERUM PROTEINS

III. Liver Injury Alone: Liver Injury and Plasma Depletion: The Eck Fistula Combined with Plasma Depletion

WM. J. KERR,[1] S. H. HURWITZ AND G. H. WHIPPLE

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

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The two preceding communications make it clear that the body can regenerate its serum proteins more rapidly and completely upon a full diet than during a fasting period. At best this reaction is sluggish even under the most intense stimulus and requires several days (see fig. 1).

We wish to submit experiments to show that liver injury tends to lessen the amount of circulating blood serum proteins and the presence of liver injury tends to retard the regeneration of serum proteins after an initial plasma depletion. Furthermore the Eck fistula liver appears to regenerate blood serum proteins more slowly than the normal or even the injured liver. The Eck fistula liver is smaller than normal, always presents evidence of degeneration (fat) and gives a subnormal functional test with tetrachlorphthalein (1).

It is to be recalled that the widest fluctuation in fibrinogen, which is an important plasma protein, will occur in parallel relationship to liver injury produced by chloroform or phosphorus (2). It is very easy to cause a wide dissociation between the curves of fibrinogen and of serum proteins. For example, a fatal chloroform poisoning will reduce the fibrinogen from 0.5 per cent to almost zero, but this may cause only a moderate fall in serum proteins of 2 per cent, perhaps from the normal level of 5 per cent to 3 per cent the day before death.

The fibrinogen is a very labile substance which seems to react to abnormal conditions with great speed and can be produced in an emergency in large amounts; for example, the fibrinogen can be completely

[1] Wm. J. Kerr, Sheldon Traveling Fellow in Research Medicine, 1916–1917, Harvard University, Cambridge, Massachusetts.

washed out of the blood but it will promptly return in a few hours (3, 4). The importance of this reaction in relation to hemorrhage is obvious as the fibrinogen forms the tough body of all blood clots.

We have seen that the serum proteins are more stable substances which cannot be regenerated in any short period of time. These proteins cannot be removed in any large amount without causing fatal shock, yet the serum proteins have some features in common with the plasma protein fibrinogen. There is some evidence in this paper to indicate an important relationship between the serum proteins and the liver, just as has been established in the case of fibrinogen. It is not too much to assume that the liver may be concerned in the production of serum protein and the maintenance of these vitally important fluid proteins at a normal level in the blood.

It is possible to make a graphic comparison of the curves of serum protein regeneration as influenced by diets, starvation and liver injury (see fig. 1). The curve of liver injury is the average of three experiments similar to table 23 with the use of chloroform or phosphorus to cause liver necrosis.

The methods have all been described in the first papers of this series. Experimental conditions were identical with those previously described.

EXPERIMENTAL OBSERVATIONS

Tables 20, 21 and 22 may be considered as a group. The normal level of the serum proteins is somewhat influenced by a fatal phosphorus poisoning, perhaps more than appears at first sight. Table 20 shows an experiment in which a normal dog was given about one-half a lethal dose of phosphorus dissolved in oil subcutaneously. The dog gave no clinical reaction but there was a slight fall in the curve of serum proteins. One week later the dog was given a lethal dose of phosphorus which was fatal in three days. There was a considerable fall in the blood serum proteins but we must not forget that this fall is probably greater than the figures indicate. Dogs poisoned with phosphorus vomit a good deal, show concentrated urine, have a tendency to hemorrhage and show a blood hematocrit indicating a relative increase in red cells. All this points to a decrease in blood volume at the expense of the plasma fraction and this means a fall in the *total serum proteins* of the body, more than is indicated by an analysis of a unit specimen of serum.

The liver of fatal phosphorus poisoning needs no detailed description. It is large and obviously fatty, specked with ecchymoses. The

microscope shows many necrotic liver cells but especially a marked fatty degeneration of the cell protoplasm in every liver lobule.

Table 21 shows the result of a lethal dose of phosphorus which killed the dog in five days. There is a progressive fall in total serum

TABLE 20

Dog 17–126. Phosphorus poisoning. Serum proteins. Mixed diet

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1917	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
March 10	5.0	2.8	2.2	1.7	12.88	
March 12	4.9	2.5	2.4	1.8	12.88	Given subcutaneously $\frac{2}{3}$ mgm. phosphorus in oil per pound body weight
March 13	4.5	2.2	2.3	1.7	12.75	
March 14	5.0	2.9	2.1	1.7	12.25	
March 15	4.8	3.0	1.8	1.8		
March 16	4.9	2.9	2.0	1.9		
March 17	4.7	2.5	2.2	1.9	12.50	No evidence of phosphorus poisoning
March 19	4.9	2.9	2.0	1.6	11.88	Given subcutaneously $1\frac{1}{4}$ mgm. phosphorus in oil per pound body weight
March 21	4.9	2.9	2.0	1.9	12.00	Serum bile-stained. Fibrinolytic test faintly positive. Not eating. Vomiting
March 22	3.8	2.0	1.8	1.6	11.75	Distinctly sick. Not eating. Vomiting. Plasma wine-colored. Clot does not form after several hours. Animal gradually failed and died in p.m. Liver necrosis and fat with multiple hemorrhages

proteins with the advance of the clinical symptoms of intoxication. The fibrinogen in both dogs was practically absent in the blood on the day before death. This explains the general hemorrhagic tendency and purpura. The blood obtained post-mortem gives a high protein reading but this has no significance because so many factors cannot be

determined. There is usually much blood concentration under suen conditions and loss of fluid to the tissues.

Table 22 gives the results of a mild chloroform poisoning which influences only slightly the level of blood serum proteins. The data of this table are to be compared with those in the two following tables

TABLE 21

Dog 17–129. Phosphorus poisoning. Serum proteins. Mixed diet

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1917	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
March 16.....	6.2	4.5	1.7	2.3	11.50	Given 1¼ mgm. phosphorus in oil subcutaneously per pound body weight
March 17.....	5.7	3.4	2.3	2.2	12.00	
March 18.....	4.8	2.5	2.3	2.1		Not eating. Vomiting. Serum bile-tinged
March 19.....	4.4	2.3	2.1	2.2	11.13	Not eating. Vomiting. Purpuric spots on neck. Serum deeply bile-tinged. Coagulation time delayed
March 20.....	3.8	0.6(?)	3.2	3.9	10.75	Vomiting. Not eating. Purpura increasing. Difficult to bleed. Serum deeply bile-stained. Serum diluted for readings. Coagulation time delayed
March 21.....	5.8	3.4	2.4	2.6		Death during night. Multiple hemorrhages. Liver necrosis. Specimen taken from heart's blood for readings. Plasma mahogany-red color. Does not clot
						Serum post-mortem

where the chloroform injury is combined with a plasma depletion. It will be noted (table 22) that the fall in total serum proteins begins on the second day after the chloroform anaesthesia and continues during the next three or four days during which period we know that the liver repair is most active. It can be estimated from all data at hand

that this chloroform anaesthesia for one and one-half hour did not cause more than one-third necrosis of each liver lobule. Furthermore, we know that such injuries can be repaired to normal in five to seven days. It will be observed that the dog was in excellent condition and gained weight during this period of repair. The notes on the diet were lost but it is practically certain that the dog was on a mixed diet.

Tables 23 and 24 are to be compared as the first shows the effects of a mild chloroform poisoning and the second of a more severe chloroform poisoning combined with a plasma depletion. Table 23

TABLE 22

Dog 16–9. Chloroform poisoning. Serum proteins

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1917	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
May 1.....	6.1	4.5	1.6	2.0		Mixed diet (?)
May 2.....	5.5	3.2	2.3	2.1		Chloroform anaesthesia 1½ hours. Well taken
May 3.....	5.9	4.3	1.6	2.4		
May 4.....	5.0	2.6	2.4	2.1	38.25	
May 5.....	4.7	2.7	2.0	2.3		
May 6.....	4.6	2.7	1.9	2.2		
May 7.....	4.4	1.8	2.6	2.6		
May 8.....	4.9	3.3	2.6	2.7		
May 9.....	4.9	2.6	2.3	2.8	40.13	
May 11.....	5.6	2.8	2.8	2.2		
May 13.....	5.3	2.9	2.4	2.7		
May 17.....	5.5	2.6	2.9	2.1		Complete recovery

also is to be compared with table 6 in the first paper of this series which shows the result of a preceding plasma depletion done upon this same dog with metabolism figures. This preceding experiment was associated with severe shock and the curve of nitrogen metabolism with and without the chloroform necrosis is very similar. We may assume that the amount of liver injury (1 hour anaesthesia) in table 23 was very slight or that the severe shock of table 6 caused as much disturbance in the animal as did the chloroform poisoning—the increase in nitrogen elimination being about the same. The influence in any case is not

very evident in the curve of serum protein regeneration. When the dog is placed on a meat diet the curve rises promptly to normal.

TABLE 23

Dog 17–130. Plasma depletion. Chloroform necrosis. Regeneration of serum proteins

DATE	SERUM PROTEINS				NITROGEN ELIMINATION	WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents			
1917	*per cent*	*per cent*	*per cent*	*per cent*	*grams*	*pounds*	
May 1	4.5	2.8	1.7	2.3	1.32	13.81	Bled 80 cc. into 8 cc. of 10 per cent citrate solution
May 2					1.18	13.06	*Plasmapharesis:* Estimated blood volume 465 cc. Four bleedings from 70 to 150 cc. each. Total 505 cc. = 109 per cent of blood volume. Reinjected 700 cc. in Locke-acacia solution
Specimen 1	5.2	3.3	1.9	2.1			
Specimen 2	3.9	2.3	1.6	2.1			
Specimen 3	3.4	1.9	1.5	2.4			
Specimen 4	2.6	1.3	1.3	2.6			
Specimen 5	2.0±	1.4	0.6±	2.7			
May	3.1	1.1	2.0	2.6	1.99	12.81	Chloroform necrosis.—1 hour light 10 cc. used
May 4	3.0	1.6	1.4	2.3	2.80	12.44	Slight hemolysis of serum
May 5	3.2	1.3	1.9	2.9	3.08	11.88	Serum slighly bile-stained
May 6					1.76	11.69	400 cc. water each day by stomach tube
May 7	3.4	1.3	2.1	2.4	1.79	11.56	
May 8	3.8	2.0	1.8	2.1	1.62	11.38	
May 9	4.2	2.2	2.0	2.3	1.46	11.13	
May 10					1.54	10.94	
May 11	4.3	2.1	2.2	2.1	1.51	10.75	
May 12	4.7	2.1	2.6	2.4	1.60	10.44	Meat diet after bleeding
May 13	4.9	2.2	2.7	2.5		11.50	Lipemic serum
May 14	4.7	2.6	2.1	2.5			
May 15	5.2	2.7	2.5	2.8		11.63	Serum slightly turbid
May 16	5.7	3.0	2.7	2.7			Serum slightly turbid
May 17	5.3	2.8	2.5	2.5		12.13	Serum slightly turbid

Table 24 brings out several points of interest. The plasma depletion was to a moderately low level but not associated with severe shock. More chloroform was given to this dog than to the dog in the

preceding experiment (table 23) and the presence of coloring matter in the serum for four days indicates a considerable amount of liver injury. The dog was clinically disturbed on the day after the anaesthesia

TABLE 24

Dog 17–186. Plasma depletion. Chloroform necrosis. Regeneration of serum proteins

DATE	SERUM PROTEIN				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1917	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
April 17	5.7	3.4	2.3	1.7	21.00	Bled 200 cc. into 20 cc. 10 per cent sodium citrate solution
April 18					20.88	*Plasmapharesis*: Estimated blood volume 713 cc. in 4 bleedings from 130 to 200 cc. each or 98 per cent of blood volume. Reinjected 910 cc.
Specimen 1	5.5	3.4	2.1	2.0		
Specimen 2	4.2	2.5	1.7	2.0		
Specimen 3	3.9	2.9	1.0	2.2		
Specimen 4	3.3	2.0	1.3	2.4		
Specimen 5	2.1	0.9	1.2	2.8		
April 19	3.0	1.9	1.1	3.1		Light chloroform anaesthesia for 1 hour. 15 cc. chloroform used. Serum normal color
April 20	2.9	1.3	1.6	2.8	20.88	Animal sick. Drinks only small amount of milk. Serum canary-yellow color (bile)
April 21	3.0	1.4	1.6	3.0	19.75	Serum slightly bile-tinged
April 22	3.0	0.9	2.1	2.6		Serum slightly bile-tinged
April 23	4.0	2.2	1.8	2.3	19.00	Serum slightly tinged
April 24	2.9	0.8	2.1	3.1		Serum normal color
April 25	4.2	2.4	1.8	2.6	19.75	Serum normal color
April 26	3.6	1.0	2.6	2.9	19.00	
April 27	4.2	2.4	1.8	3.1	18.25	Animal quiet. Discharge from eyes and nose. Distemper
April 28	3.4	2.0	1.4	3.9	18.13	Animal distinctly sick. Distemper. Serum clear
April 29	3.0	0.8	2.2	3.4		
April 30						Found dead. Broncho-pneumonia. Liver necrosis

and refused food, which indicates considerable intoxication. There is a loss of weight in spite of the fact that the dog had access to food in the cage. The amount of food eaten is not known but the dog had

bread and milk in the cage and evidently ate some of it as can be estimated from the weight curve.

The curve of serum proteins shows a slow rise toward normal followed by a terminal fall during the two days before death which was due to distemper. Autopsy showed lungs involved with bronchopneumonia, edema and congestion. Liver shows necrosis in the center of each lobule. The central three-fifths of each liver lobule is very fatty and full of large vacuolated liver cells, some fresh fibrin and leucocytes of all types. The marginal two-fifths of the liver lobule is more nearly normal. The repair of this liver necrosis is much delayed and abortive. Ten days elapsed since the chloroform injury and liver necrosis of this amount should be completely repaired within five to eight days under usual conditions. The great delay in liver repair was due in part to the intoxication and loss of appetite as well as to the intercurrent infection (distemper). The reaction on the curve of blood serum regeneration is not very profound when we consider the injury done the liver, the small food intake and the fatal outcome.

Table 25 is to be compared with the two preceding tables where the injury is due to chloroform. The type of reaction is similar and the reaction to the liver injury of phosphorus is quite definite. We are fortunate in being able to refer to other experiments upon this same dog (tables 4 and 5 in the first paper of this series) where the curve of regeneration has been influenced by fasting. The influence of phosphorus poisoning is a little more marked than the long period of fasting. During the phosphorus experiment the dog was on a liberal mixed diet but lost some weight and showed a few symptoms of intoxication. This dog received about two-thirds of a lethal dose of phosphorus. Also refer to another experiment upon this same dog (table 11). The regeneration curve of serum proteins is influenced by a meat diet and this experiment gives a perfect control to table 25.

Tables 26 and 27 are to be considered together as the experiments are similar and the results practically identical. The Eck fistula operations were performed some time before the plasma depletion as indicated in each table. The operation consists in an anastomosis between the portal vein and the vena cava by means of special clamps and a ligation of the portal vein above the anastomosis.

Table 26 gives the figures of a very successful experiment in which the plasma depletion was carried out with a minimum amount of shock. Following this exchange the dog was in excellent condition and maintained the usual weight curves. It will be seen that there is scarcely

any regeneration of serum proteins during the three days following the plasmapharesis, in spite of a liberal diet and a relatively slight plasma depletion. In a normal dog under similar conditions the regenera-

TABLE 25

Dog 16–140. Plasma depletion. Regeneration of serum proteins. Phosphorus poisoning

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1917	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
April 2	5.2	3.2	2.0	2.0	25.00	Mixed diet
April 3	5.7	3.7	2.0	2.0	25.00	Bled 180 cc. into 20 cc. 10 per cent sodium citrate solution
April 4					25.00	*Plasmapharesis:* Estimated blood volume 855 cc. Bled 970 cc. in 5 bleedings of from 100 to 210 cc. Total 113 per cent of blood volume. Reinjected 1100 cc. Non-protein nitrogen (Kjeldahl) 28.1 mgm. per 100 cc. blood
Specimen 1	5.4	3.2	2.2	1.9		
Specimen 2	4.3	2.2	2.1	1.7		
Specimen 3	3.3	1.9	1.4	2.3		
Specimen 4	3.2	1.6	1.6	2.2		
Specimen 5	2.8	1.6	1.2	2.4		
Specimen 6	1.9	0.3	1.6	2.7		
April 5	2.8	1.3	1.5	3.0	23.50	At 11.00 a.m. given $\frac{3}{4}$ mgm. phosphorus per pound body weight subcutaneously in oil
April 6	2.5	0.5	2.0	3.8	22.50	Slight vomiting
April 7	3.2	1.2	2.0	3.1	22.25	Leg wound doing well
April 9	4.0	2.2	1.8	2.4	21.50	No evidence of phosphorus poisoning
April 10	3.8	1.5	2.3	2.3	21.00	Mixed diet
April 11	3.6	1.2	2.4	2.8	21.50	
April 13	4.5	2.2	2.3	2.2	21.75	
April 14	4.2	1.6	2.6	2.8	22.25	Cloudy serum (lipemia)
April 16	4.3	1.6	2.7	2.4	23.00	
April 17	4.6	2.3	2.3	1.9	23.25	
April 18	4.9	2.9	2.0	2.0	23.38	
April 19	5.0	2.9	2.1	2.1	23.00	Serum slightly turbid
April 20	4.9	2.9	2.0	2.0	23.25	Experiment discontinued

tion would be practically complete (compare table 10 in the preceding paper). A bile fistula was performed on October 7 and the dog died on November 16 from typical Eck fistula intoxication. Au-

topsy showed that the Eck fistula was widely patent but the ligature on the portal vein was not completely occluding, a tiny opening persisting at this place. Some portal blood could gain access to the liver but it must have been small in amount and did not prevent the dog from developing the typical Eck fistula intoxication.

TABLE 26

Dog 16–115. Eck fistula. Plasma depletion. Regeneration of serum proteins

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1916	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
April 5.....					18.25	Eck fistula operation: February 22. *Plasmapharesis:* Total blood withdrawn 675 cc.; total injected, 700 cc. Total proteins reduced 44 per cent. Estimated exchange = 108 per cent of blood volume
Specimen 1...	5.5	3.9	1.6	1.7		
Specimen 2...	4.4	3.3	1.1	1.7		
Specimen 3...	3.6	2.2	1.4	1.8		
Specimen 4...	3.1	1.7	1.4	1.7	17.88	
April 6.....	3.6	2.4	0.2	1.6	17.50	Animal in good condition. Wound O.K.
April 7.....	3.5	2.1	1.4	1.9	18.00	Animal active. Bread and milk diet
April 8.....	3.4	1.3	2.1	1.7		
April 9.....	3.7	1.7	2.0	1.7		
April 10.....	3.9	2.4	1.9	1.7	18.13	
April 11.....	4.2	2.4	1.8	1.7	18.00	
April 12.....	3.8	2.1	1.7	1.7		
April 13.....	4.0	2.0	2.0	1.9	18.50	
April 14.....	3.8	2.1	1.7	1.9		
April 15.....	3.5	1.2	2.3	2.3		
April 17.....	3.4	1.5	1.9	2.1		
April 20.....	4.0	2.2	1.8	2.3	18.75	
April 24.....	4.8	3.7	1.1	2.2	19.00	
May 1.....	4.8	2.4	2.4	1.4	19.00	

Table 27 gives striking results and shows a complete absence of serum protein regeneration during the twenty-four hours after the experiment. The initial shock of the plasmapharesis was considerable in spite of the fact that the plasma exchange amounted to only 59 per cent of the estimated blood volume. The initial blood serum readings

were low and the regeneration of serum protein very slow. The dog was on a liberal diet and lost no weight. On June 28 the dog developed the typical Eck fistula intoxication. Autopsy showed a patent Eck fistula and the ligature on the portal vein completely occluding it. The liver showed the usual atrophy and central fatty degeneration which is always present in the Eck fistula dog.

Figure 1 gives a graphic comparison of curves which represent the blood serum regeneration under different conditions. Curve *D* is the

TABLE 27

Dog 16–93. Eck fistula and splenectomy. Plasma depletion. Regeneration of serum proteins

DATE	SERUM PROTEINS				WEIGHT	REMARKS
	Total proteins	Total albumins	Total globulins	Non-protein constituents		
1916	*per cent*	*per cent*	*per cent*	*per cent*	*pounds*	
April 19.....						Eck fistula operation March 10.
Specimen 1...	4.6	2.2	2.4	2.3	24.00	Splenectomy March 23. *Plasmapharesis:* Total blood withdrawn 600 cc. R. B. C. suspension injected 600 cc. Estimated exchange = 73 per cent of blood volume
Specimen 2...	4.1	1.2	2.9	3.0		
Specimen 3...	4.1	1.5	2.6	2.2		
Specimen 4...	3.4	2.1	1.3	2.6		
April 20.....	3.1	1.2	1.9	2.1	23.25	Animal very weak. 32 gm. glucose by stomach tube
April 21.....	3.7	2.3	1.4	1.7	23.25	Bread and milk diet
April 22.....	3.6	1.1	2.5	2.0	22.25	
April 24.....	3.8	2.5	1.3	2.5		
April 25.....	3.8	2.5	1.3	2.2	23.13	
April 26.....	4.1	1.6	2.5	1.7		
May 2.....	4.4	1.2	3.2	1.9	23.63	

average of seven experiments showing the serum protein regeneration on a meat diet or a full mixed diet. Curve *S* represents the average of five experiment with fasting periods after the plasma depletion. Curve *O* represents the average of three experiments in which the liver was injured on the day following the plasma depletion using chloroform or phosphorus. This curve is less regular because there are fewer experiments which in the average tend to smooth out minor fluctuations. This chart brings out the constant regeneration of about 1 per cent

total protein in the twenty-four hours following the experimental procedure of plasma depletion. In spite of food intake the curve of serum protein regeneration following liver injury is less steep than the fasting curve.

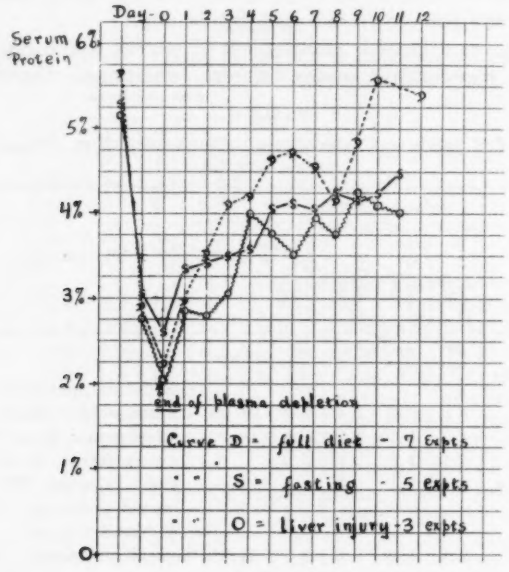

Fig. 1.

DISCUSSION

We do not wish to lose sight of the fact that the curve of blood serum regeneration is not profoundly influenced by a coincident liver necrosis (table 24) and a part of the slow regeneration may be attributed to the *low food intake* which results from the liver injury and consequent intoxication. But the amount of reserve power of the liver must not be disregarded in this complicated reaction. On the other hand we should not forget the fact that the serum proteins fall as a result of fatal liver injury.

A very important point too is that the Eck fistula liver is associated with a peculiar curve of blood serum regeneration. It will be recalled (fig. 1) that practically every experiment in plasma depletion shows a

rise of about 1 per cent during the twenty-four hours following the plasma removal. This indicates perhaps an emergency production or an emergency reserve which helps bring the serum proteins back to a safe level. The Eck fistula dog does not show this 1 per cent rise after the plasma depletion and shows a slow serum protein regeneration in spite of a generous diet. The plasma depletion can never be carried to a very low level because these dogs are very susceptible to the shock of this procedure. There is clearly much difficulty in serum protein regeneration by the Eck fistula dog. The liver of the Eck fistula dog is known to be insufficient in several capacities. These Eck fistula dogs die from peculiar intoxications associated with convulsions and in spite of considerable experimental work, the correct explanation of this intoxication has not yet been advanced.

Much experimental evidence points to the liver as being concerned in the maintenance of a normal level of blood serum proteins. The evidence here is not as convincing and striking as is the case in the plasma protein *fibrinogen* which has such an intimate relationship to liver injury and disappears almost completely with acute liver injury.

This all tends to emphasize the *stability* of the blood serum protein even under unusual conditions. We rarely find much fluctuation in the blood serum protein level in varying conditions of health or disease.

SUMMARY

Pronounced injury of the liver by means of phosphorus or chloroform will cause, or at least be associated with, a moderate fall in blood serum proteins.

Regeneration of blood serum proteins following a plasma depletion is slow at best but will be delayed by the presence of liver injury due to phosphorus or chloroform.

The Eck fistula liver is smaller than normal and is functionally deficient in some respects. Eck fistula dogs show a distinct inability to regenerate blood serum proteins following a plasma depletion. The rapid regeneration of the first twenty-four hours is especially lacking and these dogs are very susceptible to the shock of the plasma dilution.

All this experimental evidence points to the liver as concerned in maintaining the normal level of the blood serum proteins.

There is a *remarkable stability of the blood serum protein level* in widely varying conditions of health and disease.

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THE NORMAL GROWTH OF WHITE LEGHORN CHICKENS

G. DAVIS BUCKNER, R. H. WILKINS AND JOSEPH H. KASTLE[1]

From the Chemistry Department of the Kentucky Agricultural Experiment Station, Lexington

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The White Leghorn chick has been used by a number of scientists for nutritional experiments with varying degrees of success. That the White Leghorn chicken lends itself most usefully to experimentation is seen by the fact that, being a very highly nervous animal, it reacts quickly to any change of nourishment or stimulation. A value also exists in that the Leghorn breed grows rapidly, reaching maturity in less than a year. There are certain physical symptoms which are readily recognized as being caused by malnutrition or some pathological condition. Principal among these visible symptoms are the appearance of the skin on the abdomen, the condition of the vent and the anemic and drooping condition of the comb and wattles, which show only a rudimentary development if the chick does not receive the proper food during the period of growth. Each of the above named conditions would, in all probability, point to some disturbance in the respiratory or circulatory systems or to faulty diet. The drooping of the wings might mean chronic or acute digestive disorders, in the latter case the wing-drooping disappearing in a few hours. For these and other reasons the White Leghorn chick has been of great service in this laboratory, fulfilling the desired conditions of experiments now in progress.

In view of this fact it seemed most important that we definitely establish the normal curve of growth of this breed of chickens, so that an accurate control could be made of our laboratory experiments. It would naturally follow that any results obtained in the laboratory which showed a different rate of growth would be called abnormal and deductions would be made accordingly.

[1] The authors desire to express their appreciation to Prof. J. J. Hooper of the Animal Husbandry Department of this Station for generously allowing this investigation to be made at the poultry farm.

In order to cover all conditions controlling our experiments we used two lots of pure-bred White Leghorn chickens which had come from the same parent stock, each lot composed of sixty one-day-old chicks.

Lot 1 was raised under artificial conditions as follows: Two hundred eggs were hatched in an incubator and from the chicks obtained sixty were selected entirely at random and placed in a brooder-house which opened into a grass space containing 240 square feet of well-sodded earth. These chicks received numbered leg-bands and were weighed individually the day following hatching and were so weighed every week thereafter, on a torsion balance sensitive to 0.1 gram, individual records being kept. When a chick died from any cause its previous weight-record was discarded so that, at the end of twenty-eight weeks, we compiled our results from the individual records of the chicks remaining at that time. This was done in order to avoid any error in the average weight of the chicks caused by a possible loss in weight previous to death. The ration used was the standard Cornell ration which is as follows, the chemical analysis being of the particular grain mixtures fed.

Lot 2 was raised under natural conditions as follows: Two hundred White Leghorn chicks from the same parent stock as lot 1 were hatched under hens and when the chicks were one day old, sixty were selected at random and placed under four mother hens, being individually weighed at that time and weekly weighings and records made as of lot 1. The chicks were cared for by the mother hens and allowed to roam at will during the day over a large grass range and were housed at night in an ordinary coop. No special care was given them other than seeing that they were properly fed and watered. At the age of 6 weeks they were weaned from the mother hens and were placed in a pen containing an appropriate coop and with access to one-acre ranges. At this time lot 1 was removed to a pen having conditions in every way similar to those of lot 2—the feed for lot 2 being the same as that of lot 1. As soon as treading began the cockerels and pullets were separated to avoid any undue exercise on the part of the pullets, which might cause loss of weight. The mortality records of these two lots of chicks were kept to determine the variation in the vitality which might be stimulated by the freedom and natural care afforded by the mother hen during early growth.

	ANALYSIS	USED AS FOLLOWS
	Per cent	
Mixture 1 8 parts rolled oats 8 parts bread crumbs 2 parts sifted beef-scrap 1 part bone meal	Protein = 23.15 Ether extract = 5.30	1 to 5 days. No. 1, moistened with sour skimmed milk, fed five times each day. Mixture 2 in shallow tray containing a little of No.3 (dry) always before chicks, tray containing grit and charcoal. Sprouted oats once each day
Mixture 2 3 parts cracked wheat 2 parts cracked corn 1 part pinhead oatmeal	Protein = 12.30 Ether extract = 4.50	5 days to 2 weeks. No. 2 in litter twice a day. No. 3, moistened with sour skimmed milk, fed three times a day. No. 3 (dry) always available
Mixture 3 2 parts wheat bran 3 parts corn meal 3 parts wheat middlings 3 parts beef scrap (best grade) 1 part bone meal	Protein = 27.30 Ether extract = 6.20	2 to 3 weeks. As shown above except that the moist mash is given twice a day
Mixture 4 3 parts wheat (whole) 3 parts cracked corn 1 part hulled oats	Protein = 12.20 Ether extract = 4.10	4 to 6 weeks. Moist mash once a day; mixture 4 in litter twice a day; dry mash always available
Mixture 5 3 parts wheat 3 parts cracked corn	Protein = 11.30 Ether extract = 2.80	6 weeks to maturity. Nos. 3 and 5 hopper-fed. One meal a day of moist mash

Fine grit, charcoal, oyster shell and water were provided from the start.

The following table gives the average weekly weights of the chicks that remained at the close of this experiment and also the weekly mortality records of the entire lots.

TABLE 1

Average weights and mortality records of White Leghorn chicks

DATE	NUMBER OF WEEKS	LOT 1				LOT 2			
		Average weight of 1 chicken			Number surviving each week	Average weight of 1 chicken			Number surviving each week
		Cockerels	Pullets	Mean		Cockerels	Pullets	Mean	
April 18		42.1	41.1	41.6	60	42.2	40.9	41.6	60
April 25	1	72.2	69.2	70.7	55	76.8	75.2	76.0	54
May 2	2	109.7	95.6	102.6	53	108.2	101.3	104.7	54
May 9	3	161.4	140.9	151.1	53	151.2	145.0	148.1	54
May 16	4	231.6	191.7	211.6	53	209.5	198.3	203.9	54
May 23	5	292.2	248.8	270.5	53	283.8	262.4	273.1	53
May 30	6	360.9	288.3	324.6	52	374.2	328.0	351.1	53
June 6	7	442.8	354.4	398.6	52	437.8	383.6	412.2	53
June 13	8	496.9	395.6	446.2	52	580.6	490.7	535.6	52
June 20	9	537.6	409.9	473.7	51	616.6	552.8	584.4	51
June 27	10	630.9	488.7	559.8	48	729.2	648.9	689.0	51
July 4	11	674.4	506.4	590.4	48	758.4	655.5	706.9	50
July 11	12	812.9	622.2	717.5	48	926.9	775.4	851.1	50
July 18	13	902.3	682.4	792.3	48	992.7	826.8	909.7	50
July 25	14	953.2	695.1	824.1	48	1050.0	869.7	959.8	50
August 1	15	980.4	717.3	848.8	48	1106.8	898.0	1002.4	50
August 8	16	1063.2	780.9	922.1	48	1167.3	938.3	1052.8	50
August 15	17	1154.9	841.8	986.6	48	1235.6	987.2	1111.4	50
August 22	18	1252.7	889.4	1071.0	48	1282.7	1037.2	1160.0	50
August 29	19	1295.3	922.9	1109.1	47	1323.0	1045.3	1181.1	50
September 5	20	1344.8	956.2	1150.5	47	1365.4	1110.7	1238.0	50
September 12	21	1396.7	992.2	1194.4	47	1417.5	1149.2	1283.3	50
September 19	22	1430.6	1038.4	1234.5	47	1459.4	1233.1	1346.2	50
September 26	23	1442.6	1047.6	1245.1	47	1493.2	1249.4	1371.3	50
October 3	24	1462.1	1066.2	1264.1	47	1585.6	1329.9	1457.7	50
October 10	25	1480.0	1082.6	1281.3	45	1590.1	1339.0	1464.5	50
October 17	26	1528.5	1075.4	1301.9	43	1649.1	1368.7	1508.9	50
October 24	27	1560.6	1090.2	1325.4	43	1673.4	1405.4	1539.4	50
October 31	28	1594.6	1120.4	1357.5	42	1748.1	1447.5	1597.8	50

From the foregoing table and the graphs it will be seen that the average weight of one-day-old White Leghorn chicks is 41.6 grams and also that the average cockerel is 1.15 gram or 2.8 per cent heavier

than the female of similar age. The superiority in the weight of the cockerels continues throughout the growing period. Further, it is seen that at the end of twenty-eight weeks the average chick, allowed the freedom and care of a mother hen, develops a vitality which manifests itself in allowing those chicks to become 240.3 grams heavier

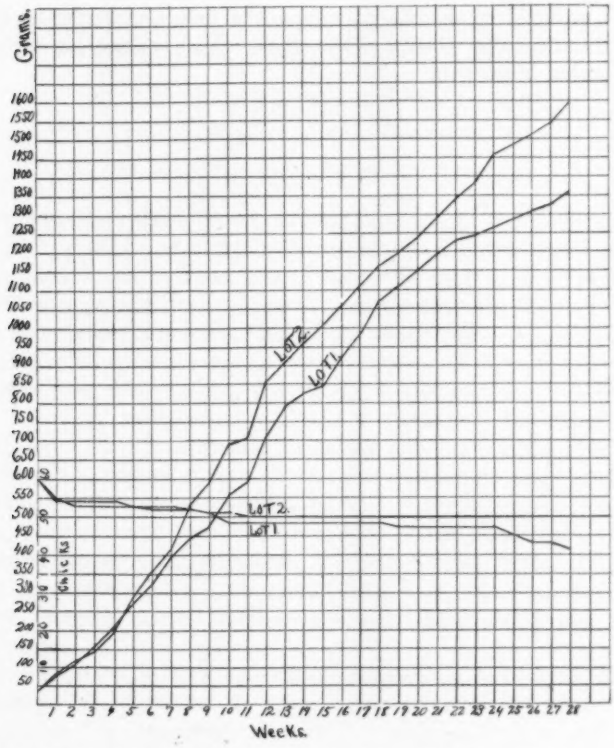

Fig. 1. Chart showing the normal rate of growth and mortality records of brooder raised (lot 1) and hen raised (lot 2) chicks.

than those raised under artificial conditions as described. This is also borne out by the mortality figures. Each lot started with sixty chicks and at the end of twenty-eight weeks ten had died from some unknown and unavoidable causes in the hen-raised lot as against eighteen deaths in the artificially raised lot.

Finally, from our results we feel justified in drawing the following conclusions:

The rate of growth of White Leghorn chicks, as shown by the graph and tabulation presented herein, closely approximates the normal.

The vitality of hen-raised chicks is superior to that of artificially raised chicks, as shown by weight and mortality.

That physical conditions governing the first few weeks of growth markedly affect the vitality of the chick, which is manifest during the entire period of growth.

SECRETIN

IV. The Number of Red and White Corpuscles in the Circulating Blood During Digestion

ARDREY W. DOWNS and NATHAN B. EDDY

From the Physiological Laboratory of McGill University, Montreal, Canada

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In a previous report (1) we have shown that in the rabbit the subcutaneous injection of secretin brings about a considerable increase in the number of red and white corpuscles in the blood stream by directly stimulating the production of new cells. Bayliss and Starling (2) in their original investigation of the pancreatic mechanism demonstrated the presence of secretin in the intestine of the rabbit as well as in that of man, the cat, dog and other animals, and concluded that it is an identical substance in all the vertebrata. According to the hypothesis of these authors and the actual demonstration of Wertheimer (3) secretin is carried in the blood during digestion and should then be at least as potent a stimulus to blood cell formation as when experimentally introduced into the circulation. The examination of the peripheral blood during digestion, therefore, ought to reveal a change in the corpuscle content comparable with our experimental findings, i.e., an increase in the number of erythrocytes and leucocytes per unit volume.

The first work in this connection appears to have been done by Moleschott (4) in 1854. He found an increase in the proportion of white corpuscles to erythrocytes which was particularly marked if the diet was rich in protein food. Hofmeister (5) in 1887 called attention to the "cellular richness" of the adenoid tissue of the intestinal wall during digestion on a meat diet and Pohl (6) went on to show that an increase in the number of white corpuscles in the circulating blood appears early in digestion and persists for several hours. Goodall, Gulland and Paton (7) and Gulland and Paton (8) went into this subject in greatest detail and found in dogs that the maximum leucocyte count occurs three to four hours after a meal. They also showed

this to be chiefly due to a very constant increase in the number of lymphocytes, contributed to much less regularly by a relative increase in the polymorphonuclear cells. They further demonstrated that the source of the leucocytosis is entirely, or almost entirely, the bone marrow in which they found an increased rate of production of white corpuscles during digestion.

Much fewer are the reported observations upon the erythrocytes during digestion. Pohl (6) found a slight diminution in their number, but he made very few determinations and did not follow the count through the entire period of digestion in any case. Buntzen and Sörensen (9) reported an increase of 8 to 25 per cent and Andressen (10) a decrease of 5 to 12 per cent in the number of erythrocytes per unit volume of blood in digestion. In estimations made before and after feeding Goodall, Gulland and Paton (7) found no change in the percentage of haemoglobin.

Källmark (11) in fasting experiments on rabbits found a slight falling off in the total leucocyte count during the fast and, as the most marked change in the differential count, a relative diminution of the lymphocytes. When the animals were again fed the total leucocyte count and the relative proportion of lymphocytes both increased above the original level. If the rabbits were allowed plenty of water he noted no change in the number of erythrocytes. According to Cabot (12) "food calls forth a greater leucocytosis in proportion as it is a novelty to the stomach." Rogers (13) and Swirski (14) in their work on the hunger contractions of the rabbit's stomach found that, even if the rabbits were put in a wire-bottomed cage, when all food was withheld they would eat their excreta and their stomachs would remain continuously partly filled; but if the animal's head was suitably fastened, the stomach completely emptied itself in about twenty-four hours. Therefore, the slight changes observed by Källmark may possibly be explained by his apparent failure to insure a total fast in his rabbits in some such manner.

We have been unable to find any record of blood counts during digestion in rabbits which have been made to endure a total fast. In view of this fact it seemed worth while to undertake such an investigation of the red and white corpuscles.

For this purpose apparently normal rabbits were selected and placed in a contrivance which might be aptly described as a pillory. This consisted of a box partitioned into two compartments. The partition was in two halves, an upper and a lower, in each of which was a rounded

opening. These together made an ovoid aperture through which passed the neck of the rabbit. When in use the rabbit's head projected on one side of the partition while his body was on the other side. With the animal thus fixed an absolute fast was readily maintained. Each rabbit was kept in this box forty to forty-four hours without food but was given as much water as it would drink.

At the end of the fasting period both the red and white corpuscles were counted. The animal was then released and put into a cage where it had free access to food and water. The food in every case consisted of green cabbage and oats. The rabbit always began eating at once and ate more or less continuously all day. Erythrocyte and leucocyte counts were made hourly for the six hours following the beginning of the feeding. As in our previous work the blood was obtained from the ear of the rabbit. The diluting fluids used were 0.5 per cent acetic acid for the white corpuscles and physiological saline solution for the red corpuscles. The counts were made in the usual manner with the Thoma-Zeiss apparatus.

We have made in all ten determinations, the results of which are summarized in tables 1 and 2. These show an average increase of 76.78 per cent in the white corpuscles and of 18.65 per cent in the red corpuscles. The maximum leucocyte count occurred 3.2 hours, and the maximum erythrocyte count 1.9 hours after the feeding started.

To what extent are these changes in the corpuscle content of the blood during digestion in the rabbit comparable with the effect of the experimental administration of secretin? The subcutaneous injection of 1 cc. of secretin solution (10 mgm. of a dried acid extract) per kilogram of body weight is capable of causing in the rabbit an increase in the red corpuscles per unit volume of blood of 17.07 per cent, appearing in 50 minutes and persisting 63.33 minutes. The same dose will bring about an increase in the white corpuscles per unit volume of 44.2 per cent, appearing in 51 minutes and persisting 78.5 minutes (15). This experimental effect of secretin is less, appears more quickly and persists for a shorter time than the findings in digestion noted above. Assuming that secretin is responsible for the latter one would expect such differences. Our experimental solution must necessarily be less active than the secretin entering the blood during digestion; a certain delay must ensue after the feeding starts before food begins to leave the stomach and secretin to be formed; also the formation of secretin and its entrance into the circulation is presumably continuous though not uniform throughout digestion once the process is started.

As mentioned above Gulland and Paton (8) determined that the source of the digestion leucocytosis is an increased rate of production of white corpuscles by the bone marrow. We have demonstrated

TABLE 1

Red corpuscles during digestion in the rabbit

ANIMAL	COUNT BEFORE FEEDING	1ST HOUR	2D HOUR	3D HOUR	4TH HOUR	5TH HOUR	6TH HOUR	PERCENTAGE INCREASE	MAXIMUM IN
									hrs.
1	5,920,000	5,788,000	6,504,000	5,456,000	5,920,000	5,888,000	5,408,000	9.86	2
2	6,584,000	7,360,000	7,712,000	6,920,000	6,320,000	6,064,000	6,368,000	17.13	2
3	5,968,000	5,624,000	5,464,000	6,800,000	7,136,000	4,960,000	5,352,000	19.56	4
4	5,756,000	7,040,000	6,144,000	6,544,000	6,208,000	6,960,000	6,378,000	22.30	1
5	5,488,000	6,320,000	6,880,000	5,896,000	6,008,000	5,600,000	6,192,000	25.36	2
1	5,536,000	6,736,000	5,784,000	4,976,000	6,176,000	5,886,000	5,320,000	21.67	1
2	6,032,000	7,328,000	6,288,000	5,792,000	6,304,000	5,360,000	5,304,000	21.48	1
6	5,736,000	6,128,000	6,512,000	4,800,000	5,600,000	6,092,000	5,720,000	13.52	2
7	6,240,000	6,560,000	6,880,000	6,592,000	5,936,000	5,864,000	5,896,000	10.25	2
8	6,200,000	6,960,000	7,776,000	7,008,000	5,876,000	5,916,000	6,164,000	25.41	2

TABLE 2

White corpuscles during digestion in the rabbit

ANIMAL	COUNT BEFORE FEEDING	1ST HOUR	2D HOUR	3D HOUR	4TH HOUR	5TH HOUR	6TH HOUR	PERCENTAGE INCREASE	MAXIMUM IN
									hours
1	8,000	22,000	16,600	16,000	20,000	16,000	12,000	175.00	1
2	10,000	15,000	13,000	12,700	16,000	10,000	6,600	60.00	4
3	14,000	15,000	24,000	18,600	13,000	21,800	15,000	71.42	2
4	15,000	18,000	19,000	22,400	20,000	20,700	12,600	49.33	3
5	23,400	24,000	28,000	18,000	25,000	20,000	17,600	19.65	2
1	11,000	12,000	15,000	16,000	14,200	19,400	14,000	76.36	5
2	9,600	11,500	13,000	17,500	16,800	17,000	9,000	82.29	3
6	16,000	14,000	22,100	19,000	35,000	18,400	14,000	118.74	4
7	13,200	12,500	21,600	18,000	22,000	14,000	12,000	66.66	4
8	12,400	13,200	16,000	17,000	18,400	16,400	14,500	48.38	4

(1) that secretin acts by directly stimulating the production of both red and white corpuscles by the bone marrow. May it not be that the increased activity of the bone marrow in digestion is the result of the direct stimulating action of secretin?

Additional evidence of the change in the corpuscle content of the blood during digestion being due to secretin is afforded by the differential leucocyte count. The findings of Goodall, Gulland and Paton (7) and of Källmark (11) indicate that, while the change in the leucocyte count during digestion is participated in by all the varieties of white cells, the greatest increase occurs in the lymphocytes. We have previously reported (16) the differential leucocyte count after the administration of secretin as compared to the normal; there also, while all forms are increased in number, the lymphocytes show the greatest relative increase.

CONCLUSIONS

1. During digestion there occurs an increase in the number of both the red and white corpuscles per unit volume of blood.

2. These changes are comparable with the effects produced by the experimental administration of secretin.

3. The source of the increased corpuscle content of the blood both in digestion and after the administration of secretin is an increased rate of production of new cells by the bone marrow.

4. The similarity between the differential leucocyte counts in digestion and after the administration of secretin is additional evidence of secretin being the cause of the increase in the number of corpuscles during digestion.

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POSSIBLE PERIODIC VARIATIONS IN THE EXTENT OF THE KNEE-JERK IN WOMEN

JESSIE L. KING

From the Department of Physiology, Goucher College, Baltimore

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The numerous attempts made to determine by physiological and psychological tests whether there is a monthly cycle in the life-processes of women have failed to give definite positive results, except as concerns the slight constant variations in pulse and temperature observed by many workers (1). Recent metabolism experiments add further evidence against the old theory of a rhythmical movement for they show that there is no pronounced periodic variation in the metabolism of either nitrogen, phosphorus or calcium (2), (3), (4).

The rise in temperature immediately preceding and accompanying the onset of menstruation may account for the feeling of discomfort—a certain amount of irritability and mental depression—which is frequently felt by normal women not troubled by dysmenorrhea. Yet it seems possible that there may be some variation in the tone of the nerve centers.

With the hope of throwing light on the question, at Doctor Howell's suggestion, I made a series of observations on a group of young women, using the knee-jerk as the physiological index of tonus. It is well recognized that the extent of the knee-jerk depends on the activity of the nerve centers, increasing or diminishing by whatever increases or diminishes the tone of the central nervous system (5), (6), (7), (8). Further the reflex is easily and constantly elicited from practically all normal individuals. An experiment in which amplitude alone is employed as an index of variation suffers under certain disadvantages for, as Lombard, Bodwitch and Warren and others have shown, although the amplitude is in general proportional to the blow, various interesting and obscure conditions may cause remarkable variations (7), (9). Dodge (6) warns especially against using amplitude "as an indicator of the variations of any one selected factor." It was considered, however, that averages of a large number of readings taken over periods of several weeks might be at least suggestive of general tendencies.

METHOD AND OBSERVATION

The apparatus employed was that used for classroom demonstrations at the Johns Hopkins Medical School. It was so arranged with pulleys and weights that a cord attached to the heel pulled a pointer by which the extent of the kick was recorded on a kymograph. The taps were administered by an electrical hammer. The technique, to be sure, was far from ideal but the conditions of the experiment were kept as constant as possible. For each individual the observation hour, the weight pulled and the arc through which the hammer fell were the same. From ten to fifteen contractions were recorded each day at regular intervals, the height measured in millimeters and the average taken. While the records were being made the subject closed her eyes and was asked to think of something definitely apart from the experiment. Although it became a matter of routine, no doubt such things as a desire to be through with the experiment or disquiet regarding a coming class or examination sometimes acted as a modifying influence.

Fourteen young women, college students and instructors, served as subjects. The ages ranged from eighteen to thirty-four years. They were in normal health and the menstrual periods caused no interruption in their regular college duties. Of the fourteen, five were finally discarded—one because of a systematic failure to give a response sufficiently strong to be accurately recorded, two others were so sensitive that the slightest tap caused a kick often too extensive to be registered with the apparatus used, while in the other two interruptions in the records at critical periods unfortunately decreased their value. On the other hand one subject (I) served as subject for three different periods, several months intervening between each; another (VI) at two periods with a four months interval; while in a third (IX) two years intervened between the observations. The records of these nine women included from one to four menstrual periods—thirty-three in all, their duration varying from four to seven days. The four days preceding and following the period have been uniformly considered as the pre- and post-menstrual intervals and the remaining days of the cycle as the intermenstrual phase.

Both morning and afternoon records were made from three subjects with the same general results but since those taken in the morning tended to be more regular, only a morning hour was adopted for the others of the group. The amplitude of the contractions for an obser-

vation period always varied—sometimes as much as from 60 to 70 mm. —though in general the tendency would be for all to be either high or low. Unaccountable changes naturally occur in the averages from day to day, for example taking a few successive days from the record of an intermenstrual period the averages in millimeters are 85, 93, 106, 53, 33, 35, 19. Curves were plotted from the daily records of each subject but the mathematical curves with fewer irregularities were more easily interpreted. Figure 1 gives such for subject II; above the daily record a summary of the days of each phase is given.

A consideration of the data for each individual has led to the preparation of the following summaries.

In subject I a rise occurs during four of the menstrual periods, depression precedes the onset and follows the cessation. In the other three the maximum increase does not appear until the postmenstrual phase.

In subject II two complete cycles were covered, a third cycle lacked the premenstrual and a fourth, the postmenstrual phase. In each instance a premenstrual rise is followed by a period of depression (fig. 1).

Two cycles in subject III show a premenstrual depression and a rise during the period but in a third the maximum is not attained until its cessation.

The record of IV shows nothing regular or significant except a slight depression during the two menstrual periods followed in one by a rise and in the other by continued depression.

In subject V a marked menstrual rise is preceded and followed by depression in the one cycle covered.

The record of VI, including almost five cycles, failed to give constant or significant results.

In VII the one outstanding point was the definite rise immediately following the cessation of the two periods.

A premenstrual rise is followed in VIII by a gradual decline which continues into the postmenstrual phase.

The first series of observations on IX, including four cycles, show nothing of significance but in a second series a premenstrual rise precedes three periods and in a fourth a slight increase accompanies it.

The general trend of the curves of these nine subjects may be more readily compared by examining figure 2, in which for each the averages of the observations made during all the days of the premenstrual, menstrual, postmenstrual and intermenstrual phases are shown. Summarizing the above results—a significant change in the amplitude

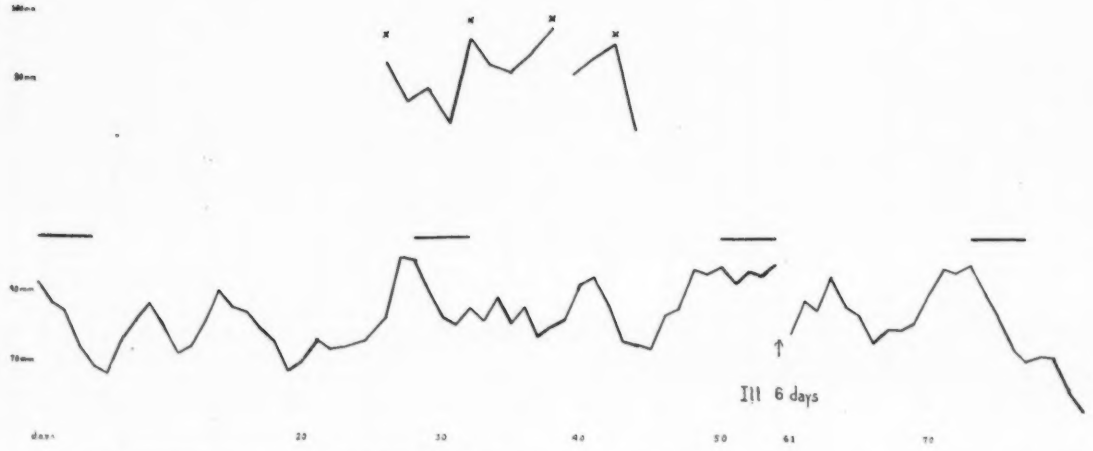

Fig. 1. Mathematical curve plotted from daily observations of the knee-jerk made on subject II. Each point is the average of from ten to fifteen readings. The menstrual phases are included under the horizontal lines. The shorter curve above represents the averages of the observations for all the days of each phase. The crosses indicate the menstrual averages.

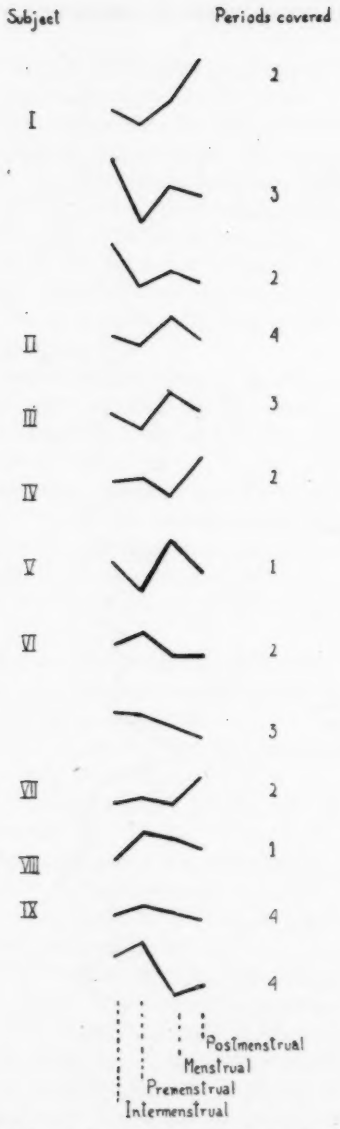

Fig. 2. Curves plotted from the averages of all the days included in the four phases for each of the nine subjects. (These curves are drawn on a smaller scale than those of figure 1.)

of the knee-jerk is shown in twenty-three instances, occurring in eight individuals. Eleven of these show a menstrual rise, six a rise immediately preceding the onset and six a rise at its close. Ten periods in four individuals show practically no change. In some instances a period of decided depression precedes the menstrual onset and in the majority of cases depression again occurs after the high point is reached, the tendency then being to approach an intermenstrual average which falls between the two extremes.

CONCLUSION

If the knee-jerk may be taken as a physiological index of the tone of the nerve centers, the evidence points in the direction of the following conclusions—that there is a period of hyperexcitability immediately preceding, accompanying or following menstruation and that there is then a tendency toward a fall and an approach to an intermenstrual average.

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